The Role of Viral Load in the Pathogenesis of HIV-2 Infection in West Africa

Thesis

UNRESTRICTED

THE ROLE OF VIRAL LOAD IN THE PATHOGENESIS OF HIV-2 INFECTION IN WEST AFRICA

BY

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Dedication

This thesis is dedicated to my parents.

Thanks to the people of West Africa.

(Caio, Guinea-Bissau. March 1997).

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Abstract.

The role of Human Immunodeficiency Virus type 2 (HIV-2) viral load has been studied in West Africa in order to understand how this infection differs from HIV-1 and why the majority of HIV-2 infected individuals live for long.

Using a polymerase chain reaction (PCR) based-assay, it was found that the level of HIV-2 provirus was surprisingly high in HIV-2 seropositive people who live long without sign of immunosuppression; in contrast the rate of HIV-2 replication reflected by RNA viral load was found low. However, HIV-2 replicates to a high titre in some HIV-2-infected people whose disease progresses rapidly to death. Longitudinal follow-up demonstrated that base-line HIV-2 RNA viral load predicts the rate of $CD4^+$ T cell decline and death but the level of proviruses is a less sensitive predictor.

Cytotoxic T-lymphocyte activity (CTL) against HIV-2 structural proteins, especially Gag, was found in most HIV-2 infected people and was inversely correlated with HIV-2 proviral load. Strong CTL activity was consistently found in people who did not have progressive disease, suggesting that CTL play an important role in controlling HIV-2 replication.

Co-infection with human T-lymphotropic virus type I (HTLV-I) occurred frequently in HIV-2-infected people in a village in Guinea-Bissau but did not seem to enhance HIV-2 viral load. However an increase in HIV-2 viral load was found in an HIV-2-infected individual with malaria parasitaemia, suggesting that malaria infection enhances HIV-2 viral load.

The majority of people who are dually seroreactive for both HIV-1 and HIV-2 are infected with both viruses but some subjects are dually seroreactive because antibodies against one

type cross-react with the other type of HIV. These cross-reactive antibodies may be distinguished by antibody dilution analysis. Other dually seroreactive people harbour an undetectable level of HIV-2 provirus and HIV-2 proviral load tended to be low in advanced disease while HIV-1 proviral load was high.

List of Contents.

Title page i
Dedication ii
Acknowledgements iv
Abstract vi
List of Contents viii
List of Figures xiii
List of Tables xv

Chapter 1. General Introduction 1

1.a. General review of HIV-2 research 1
1.a.1. History of HIV-2 discovery 1
1.a.2. HIV-2 Epidemiology 2
i. Geographic distribution 2
ii. HIV-2 epidemic in The Gambia and surrounding countries 2
iii. Age-specific prevalence of HIV-2 5
iv. Transmission of HIV-2 5

1.a.3. Clinical features and natural history of HIV-2 infection 5

1.a.4. HIV-2 virology 8
i. Structure of HIV-2 8
ii. Significance of the vpx and the vpu genes 8
iii. Genotypic and phylogenetic analysis of HIV-2 8
iv. Regulation of HIV-2 gene expression 11
v. In vitro biological characteristics of HIV-2 15
vi. Cell tropism of HIV-2 16

1.a.5. Host-immune response to HIV-2 17
i. Humoral immunity 17
ii. Cell-mediated immunity 18

1.b. Review of serodiagnosis of HIV-infections in West Africa 19

1.c. The role of HIV-1 viral load in HIV-1 pathogenesis 20
1.c.1. HIV-1 pathogenesis 20

1.c.2. HIV-1 virus population dynamics *in vivo* 21

1.c.3. Measurement of HIV-1 viral load 24
i. Viral load in peripheral blood versus lymphoid tissue 24
ii. Methods used to measure viral load 24

1.c.4. HIV-1 viral load as a marker of disease progression 27

1.c.5. HIV-1 long-term non-progressors 27

1.d. The aim of this thesis 28

Chapter 2. Materials and Methods 30

2.a. Patients and materials 30
2.a.1. A community-based study in a rural village in Guinea-Bissau 30
2.a.2. A hospital-based study at the MRC hospital in The Gambia 32
2.a.3. A community-based perinatal transmission study in The Gambia 34
2.b. Serological diagnosis 34
2.b.1. Serological diagnosis of HIV infection 34
2.b.2. Titration of anti-HIV-1 and anti-HIV-2 antibodies 35
2.c. PCR-based diagnosis 35
2.c.1. Extraction and quantification of cellular DNA 35
2.c.2. Qualitative PCR for diagnosis of HIV infection 36
2.c.3. Qualitative PCR for diagnosis of HTLV-I 37
2.c.4. Quantitative DNA PCR 40
i. Quantification of HIV-1 and HIV-2 proviral DNA 40
ii. Quantification of HTLV-I proviral DNA 43
2.c.5. Quantitative RNA PCR 43
i. Extraction and reverse transcription of HIV RNA 43
ii. Detection and quantification of HIV-2 cDNA 44
2.d. Cytotoxic T-lymphocyte assay 49
2.d.1. Preparation of target cells 49
2.d.2. Preparation of effector cells 49
2.d.3. ^{51}Chromium release assay 49
2.e. Miscellaneous methods 50
2.e.1. CD4/CD8 subset analysis 50
2.e.2. Examination of blood film 51
2.e.3. Other laboratory tests 51

Chapter 3. A community-based study of HIV-2 proviral load in a rural village in West Africa 52
3.a. Introduction 52
3.b. Results 53
i. Study subjects 53
ii. Detection and measurement of HIV-2 proviral load 53
iii. Proviral load in relation to age 54
iv. Proviral load in relation to co-infections 57
v. Proviral load in relation to mortality 60

3.c. Discussion..... 60

3.d. Discussion of the 1996 follow-up study..... 63

Chapter 4. Plasma RNA viral load predicts the rate of CD4 decline and death in HIV-2 infected patients in West Africa..... 64

4.a. Introduction..... 64

4.b. Results..... 65

i. Study subjects..... 65
ii. Characteristics of subjects..... 66
iii. Base-line viral load in relation to CD4% slope..... 66
iv. Survival analysis..... 70
v. Longitudinal observation of RNA and DNA viral load..... 72

4.c. Discussion..... 75

Chapter 5. HIV-2-specific Cytotoxic T-cell activity is inversely related to proviral load..... 77

5.a. Introduction..... 77

5.b. Results..... 78

i. Study subjects..... 78
ii. HIV-2 specific CTL activities..... 78
iii. Comparison of CTL activities and HIV-2 proviral load..... 81

5.c. Discussion..... 84

5.e. Longitudinal observation of HIV-2-specific CTL..... 86

Chapter 6. Effects of various co-infections on HIV-2 viral load 89

6.a. Lack of *in vivo* evidence for the enhancement of HIV-2 infection by HTLV-I..... 89

6.a.1. Introduction..... 89

6.a.2. Results..... 91

i. Study subjects..... 91
ii. Characteristics of subjects..... 91
iii. HTLV-I viral load among HIV-2 positive individuals and HIV negative individuals..... 93
iv. Comparison between HTLV- viral load and HIV-1 proviral load..... 96

6.a.3. Discussion..... 98

6.b. The impact of *Plasmodium falciparum* parasitaemia on HIV infection. 101

6.b.1. Introduction..... 101

6.b.2. Results..... 102

i. Study subjects..... 102
ii. Prevalence and density of Plasmodium falciparum parasitaemia..... 102
iii. HIV viral load..... 102
iv. Changes in $CD4^+$ cell count..... 106

6.b.3. Discussion..... 108

Chaper 7. PCR analysis of HIV-1 and HIV-2 proviruses in dually seroreactive Individuals..... 110

7.a. Comparison between qualitative PCR and serodiagnosis..... 110
7.a.1. Introduction..... 110

7.a.2. Results
i. Study subjects..... 111
ii. Qualitative PCR versus competitive ELISA and PeptiLav..... 111
iii. Qualitative PCR versus HIV-1 and HIV-2 antibody titres..... 113
iv. Longitudinal observation of HIV-1 and HIV-2 provirus In dually infected individuals..... 116

7.a.3. Discussion..... 118

7.b.Comparison of HIV-1 and HIV-2 proviral load in dually infected individuals..... 121
7.b.1. Introduction..... 121

7.b.2. Results
i. Study subjects..... 122
ii. Specificity of HIV-type specific quantitative PCR..... 122
iii. Comparison of HIV-1 proviral load and HIV-2 proviral load among dually infected individuals..... 123
iv. Proviral load in relation to $CD4^+$ cell count..... 126
v. HIV-proviral load in subjects who became dually infected during follow-up..... 126

7.b.3. Discussion..... 130

Chapter 8. General Discussion..... 133

8.a. The role of HIV-2 viral load in the pathogenesis of HIV-2 infection..... 133

8.b. Why do HIV-2-infected individuals live for long?..... 133

8.c. Why is the level of HIV-2 RNA virus in plasma low?..... 134

8.d. What factor determines the low HIV-2 RNA viral load?..... 138
i. Host-factors determining the low level of HIV-2 viral replication..... 138
ii. Viral factors determining the low rate of HIV-2 replication..... 139

8.e. Other possible explanation for the better prognosis of HIV-2 infection..... 141

8.f. Summary 141

References 144

Appendix 1: Supplement to Chapter 7 193

Appendix 2: Publications arising from this thesis 196

List of Figures.

1.1. Number of HIV seropositive patients who were diagnosed at the MRC hospital...3

1.2. Genomic organisation of HIV-2 and the major virion-encoded proteins..... 9

1.3. Comparison of the enhancers in the U3 domains of the HIV-1 and HIV-2 LTRs.. 12

1.4. Schematic diagram of HIV-1 population dynamics *in vivo*..... 23

2.1. Map of West Africa..... 31

2.2. Map of The Gambia..... 33

2.3. Standard curves for quantitative DNA PCR assays..... 42

2.4. Standard curve for quantitative RNA PCR assay..... 47

3.1. HIV-2 proviral load and CD4% in 104 villagers..... 55

3.2. HIV-2 proviral load in three age groups..... 56

3.3. Restriction enzyme analysis of HTLV-I/II *tax/rex* PCR products..... 58

4.1. Comparison between HIV-2 viral load and $CD4^+$ cell decline.....69

4.2. Kaplan-Meier survival analysis of 38 HIV-2-infected subjects according to RNA and DNA viral load..... 71

4.3. Profiles of CD4%, RNA, and DNA viral load of 5 fast-progressors..... 73

4.4. Profiles of CD4%, RNA, and DNA viral load of 4 long-term slow/non-progressors.....74

5.1. Correlation between HIV-2 proviral load and HIV-2-specific CTL activities..... 83

5.2. HIV-2 Gag CTL in the first (I), second (II), and third (III) year of the study..... 88

6.1. Histogram of HTLV-I proviral load in HIV-2-infected and HIV-uninfected subjects..... 94

6.2. Relation of HTLV-I proviral load to HIV-2 proviral load..... 97

6.3. RNA and DNA viral load in samples from four patients with and without *P.f.* parasitaemia..... 105

7.1. Anti-HIV-1 and anti-HIV-2 antibody titres of dually seroreactive subjects according to PCR results..... 114

7.2. HIV-1 and HIV-2 proviral load among dually infected patients..... 125

7.3. HIV-1 proviral load and CD4% in dually infected individuals..... 127

7.4. HIV-2 proviral load and CD4% in dually infected individuals..... 128

8.1. HIV-2 population dynamics 1 *in vivo*..... 136

8.2. HIV-2 population dynamics 2 *in vivo*..... 137

List of Tables

1.1. The prevalence of HIV-1, HIV-2 and dual seroreactivity in The Gambia..... 4

2.1. Primer sequences for nested PCR for HIV-1, HIV-2, and HTLV provirus detection..... 39

2.2. Primer sequences for RT reaction and nested PCR for HIV-2 viral RNA detection..... 48

3.1. Effects of co-infection with active syphilis, malaria and HTLV-I on HIV-2 proviral load..... 59

4.1. Characteristics of study patients when grouped by RNA or DNA viral load..... 67

4.2. Characteristics of 5 long-term slow/non-progressors and 4 fast-progressors..... 68

5.1. HIV-2-specific lysis at various E:T ratios to Gag, Pol, and Nef proteins and its relation to CD4%, proviral load and clinical status..... 79

5.2. Correlation between HIV-2-specific lysis to different gene products and HIV-2 proviral load or CD4%..... 82

6.1. The sex ratio, mean age and mean CD4% in subjects according to HTLV-I and HIV-2 infection..... 92

6.2. Comparison of limiting dilution analysis using two different methods of sample dilution..... 95

6.3. Geometric means (95% C.I.) of RNA viral load and DNA viral load between the dry season and the wet season..... 104

6.4. Mean (95% C.I.) $CD4^+$ cell count in the dry season and in the wet season..... 107

7.1. Results of nested PCR in subjects testing dually seropositive by PeptiLav and routine type-specific ELISA..... 112

7.2. Results of nested PCR in subjects testing dually seropositivity by PeptiLav and type specific ELISA at dilution of 1:10..... 115

7.3. Longitudinal observation of HIV-1 and HIV-2 provirus among 10 dually seropositive subjects..... 117

7.4. The geometric mean (95% C.I.) of proviral load among dually infected patients..... 124

7.5. The geometric mean of proviral load among seroconverters and the hospital patients..... 129

8.1. Summary of host-virus relations in HIV-1 and HIV-2 infections..... 142

8.2. Summary of important research areas of host and viral factors in HIV-2 infection..... 143

Chapter 1.

General Introduction.

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV). The epidemic of this virus in Central and East Africa started in the late 1970's and early 1980's (Clumeck et al., 1984; Piot et al., 1984; Van de Perre et al., 1984; Brun-Vezinet et al., 1984; Serwadda et al., 1985; Kreiss et al., 1986; Quinn et al., 1986). It was well established before the virus was discovered and related to the disease (Barre-Sinoussi et al., 1983; Popovic et al., 1984). A new United Nations report in 1997 has estimated that 20,800,000 people are living with HIV in subSahara Africa which amounts to about two thirds of the 30.6 million people living with the virus in the world. The HIV virus, which has spread in pandemic form in Africa and in other parts of the world, and has caused AIDS in the vast majority of infected people is HIV type 1 (HIV-1). On the other hand there is second type of HIV (HIV-2) which occurs on a smaller scale and is of considerably less virulence. Therefore it is believed that an understanding of real differences between the two types of HIV is important since this information may provide a deeper understanding of the nature and pathogenesis of both infections. Thus this thesis focuses on HIV-2 infection.

1.a. General review of HIV-2 research

1.a.1. History of HIV-2 discovery

HIV-2 was first isolated and described in 1986 in two West African patients with the AIDS who were repeatedly negative for serum antibodies to HIV-1 (Clavel et al., 1986). Soon after more cases of AIDS with the new type of HIV were reported; clinical features were similar to those in patients with HIV-1 and all patients were from West Africa (Brun-Vezinet et al., 1987, Clavel et al. 1987).

1.a.2. HIV-2 Epidemiology

i. Geographic distribution

Subsequent epidemiological surveys have revealed that HIV-2 is mainly confined to West Africa (reviewed by De Cock et al. 1989, 1991a, 1993). HIV-2 infections were also reported outside West Africa in countries or areas which have a present or past Portuguese connection: Angola (Bottiger et al., 1988), Mozambique (Barreto et al., 1993), Portugal (Smallman-Raynor and Cliff, 1991), Brazil (Cortes *et al*, 1989), Southern India (Pfutzner *et al*, 1992). Although sporadic cases of HIV-2 infection have been reported throughout Europe and in the north America , most infections have been in persons from or previously resident in West Africa (Smallman-Raynor and Cliff, 1991; O'Brien et al., 1992).

ii. HIV-2 epidemic in The Gambia and surrounding countries

In surrounding countries such as Senegal, Guinea-Bissau, HIV-2 does not appear to be a new infectious agent since the incidence of HIV-2 did not increase during the study period (Kanki et al., 1994; Poulsen et al., 1997). In The Gambia, numbers of newly diagnosed HIV-positive patients attending the MRC hospital are shown in Figure 1.1 and indicate that the number of newly diagnosed HIV-2-positive patients remained the same whereas the number of newly diagnosed HIV-1-positive patients has increased from around 1990. Two cross-sectional community-based studies were conducted in 1988 (Wilkins et al. 1992) and 1993/5 (O'Donovan et al. 1996) and three cross-sectional surveys of commercial sex workers (CSW) were done in 1988 (Pepin et al. 1991a), 1989 (Pepin et al., 1991b) and 1992/3 (Hawkes et al., 1994). The data are summarised in Table 1.a.1 and also show that the seroprevalence of HIV-2 was constant while the seroprevalence of HIV-1 has begun to increase.

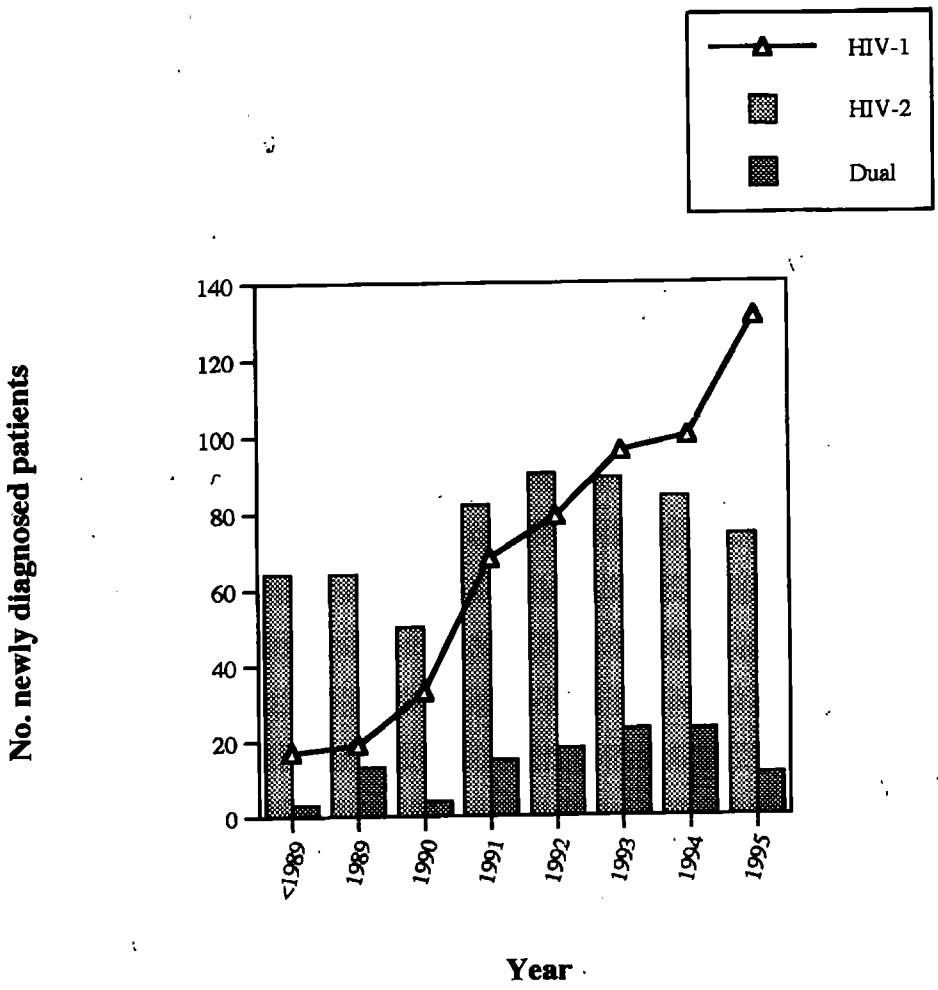

Figure 1.1. Number of HIV seropositive patients who were newly diagnosed at the MRC hospital.
(Ariyoshi et al., 1998)

	Number	HIV-1[@]	HIV-2[@]	Dual
Community-based survey				
1988*	4,228	<0.1%	1.1%	-
1993/5†	29,670	0.55%	1.15%	0.05%
CSW survey✤				
1988	355	1.7%	25.7%	1.1%
1989	241	4.6%	28.6%	2.5%
1992/3	213	13.6%	26.7%	5.6%

Table 1.1. The prevalence of HIV-1, HIV-2 and dual seroreactivity in The Gambia.
* The seroprevalence in the cluster survey was extracted from the paper(Wilkins et al. 1992); †Pregnant women attending antenatal clinic at the eight major government health centres (O'Donovan et al. 1996); ✤Commercial sex workers in 1988 (Pepin et al. 1991a), 1989 (Pepin et al., 1991b), 1992 (Hawkes et al., 1994); [@] % of HIV-1/HIV-2 dually positive individuals were added.

iii. Age-specific prevalence of HIV-2

HIV-2-infected individuals are older than HIV-1-infected individuals. A survey of pregnant women has shown that the mean age of HIV-2 seropositive pregnant women in The Gambia was 27.8 years old which was significantly older than that of HIV-1 seropositive pregnant women, being 23.6 years old ($p<0.01$) (D O'Donovan et al., 1996). The difference is clearer in the comparison of surveys in two rural communities: the peak ages of HIV-2 prevalence in a rural community in Guinea-Bissau were 45-54 years old for men and 35-44 years old for women (Wilkins et al., 1993) whereas the peak age of HIV-1 prevalence in a rural community in Uganda was 25-34 years old for both men and women (Mulder et al., 1994). This difference may be because HIV-2-infected individuals live longer than HIV-1-infected individuals and/or because the risk of HIV-2 infection is higher in the older individuals (Aaby et al., 1996; Poulsen et al., 1997).

iv. Transmission of HIV-2

Risk factors for HIV-2 infection were studied in West Africa and found to be similar to those for HIV-1 infection, suggesting that the main route of HIV-2 transmission is heterosexual (reviewed by De Cock et al. 1991a). Mother-to-child transmission of HIV-2 also occurs (Morgan et al., 1990; Adjorldo-Johnson et al., 1994; Whittle et al., 1997). Nevertheless the rate of transmission is significantly lower in HIV-2 infection than in HIV-1 infection in both heterosexual (Kanki, et al. 1994) and mother-to-child routes: the rate of perinatal transmission in HIV-1 was reported to be about 15% to 35% (reviewed by Newell, 1993) whereas that in HIV-2 was found to be 0% to 4% (Poulsen et al., 1992; Adjorldo-Johnson et al., 1994; Whittle et al., 1997).

1.a.3. Clinical features and natural history of HIV-2 infection

It was initially believed that the clinical picture of HIV-2-induced AIDS was similar to that

of HIV-1-induced AIDS (Clavel et al., 1987; Brun-Vezinet et al., 1987). Autopsy results from Abidjan have also shown the pathologies of patients who died with HIV-2 were similar to those with HIV-1 except for three conditions, all more frequent in HIV-2 deaths: i) severe multi-organ cytomegalovirus infection, ii) HIV encephalitis, iii) cholangitis. (Lucas et al., 1993). More recently it has been noticed that among patients attending the MRC Hospital, HIV-1-infected patients were 12.4 times more likely to have Kaposi's sarcoma than HIV-2-infected patients even after adjusting for $CD4^+$ cell count and age (Ariyoshi et al., 1998). However cervical cancer precursor lesions were reported to be similarly associated with both HIV-1 and HIV-2 (Langley et al., 1996). More detail clinical analysis of HIV-2-induced AIDS as compared with HIV-1-induced AIDS is necessary.

Although HIV-2 was first noticed in symptomatic patients who were diagnosed as AIDS (Clavel et al., 1986, 1987; Brun-Vezinet et al., 1987), it has been widely believed that HIV-2 is less pathogenic than HIV-1. Cross-sectional studies showed that the mean $CD4^+$ cell count among HIV-2-infected individuals was significantly lower than HIV-uninfected controls but higher than that in HIV-1-infected individuals (Lisse et al., 1990; Pepin et al., 1991b; Kestens et al., 1992). Also survival analysis of HIV-2 infected individuals at the MRC hospital indicated that HIV-2-infected patients live longer than HIV-1-infected patients (Whittle et al., 1994). The most striking evidence for the better clinical course of HIV-2 infection has derived from cohorts of community-based populations or seroconverters. Two community-based surveys in Guinea-Bissau have shown that the mortality rate ratio between HIV-2-infected and HIV-uninfected individuals was very low, from two to four fold depending upon the length of follow-up (Poulsen et al. 1989, 1997; Ricard et al. 1994). Intriguingly the longer the subjects were followed up the lower the mortality rate ratio (Poulsen et al., 1997). This was in contrast to a similar study in HIV-1 infection in a rural area of Uganda where the mortality rate ratio was 20 among the age

group corresponding to the HIV-2-cohort (Mulder et al., 1994; Poulsen et al., 1997), though a formal comparison between the mortality rate among HIV-1-infected individuals and HIV-2-infected individuals in the same geographical area has not been made. A cohort of a limited number of HIV-1 and HIV-2 seroconverters among CSWs in Senegal has been described. This cohort demonstrated that the time taken to develop AIDS or an abnormal $CD4^+$ cell count was longer in HIV-2 than HIV-1-seroconverters; the study showed that 33% of 31 HIV-1-seroconverters developed AIDS within 5 years whereas none of 32 HIV-2-seroconvertors developed AIDS within this period (Marlink et al., 1994). The absence of HIV-2 fast progressors in their cohort may be due to the small number of subjects or to the short period of follow-up. A report from Europe has described a HIV-2 seroconverter who had a rapid decline in $CD4^+$ cell count and progressed to AIDS within 36 months (van der Ende et al., 1996). Fast-progressors with HIV-2 infection are also described in Chapter 4. Therefore there is a dichotomy in HIV-2 disease progression; certain individuals when infected with a particular strain of HIV-2, have as poor clinical prognosis as that of HIV-1. An understanding of why some HIV-2-infected individuals progress fast is equally important in order to answer the question why the prognosis of HIV-2 is, in general, better than HIV-1.

1.a.4. HIV-2 Virology

In this section, virological and molecular biological studies of HIV-2 are reviewed.

i. Structure of HIV-2

The morphological structure of HIV-2 as observed by electron-microscopy is similar to HIV-1 (Clavel et al. 1986). The organisation of the HIV-2 genome, identifying the structural, regulatory and accessory genes and their major gene products, is shown in Figure 1.2. The genomic structure of HIV-2 is also similar to HIV-1 (Guyader et al. 1987) except that the *vpu* gene is present only in HIV-1/SIVcpz whereas the *vpx* gene is present only in HIV-2/SIV (Cohen et al., 1988; Henderson et al., 1988).

ii. Significance of the vpx and the vpu genes

The *vpx* gene may have originated from the *vpr* gene as *vpr* and *vpx* share considerable sequence similarity (Tristem et al., 1990, 1992). In SIV model, both *vpr* and *vpx* are required for pathogenesis (Gibbs et al., 1995). However the role of *vpx* gene in relation to the non-pathogenic properties of HIV-2 has not been clarified. It is also important to study functions of the *vpu* gene as it may contribute to the increased virulence of HIV-1 relative to HIV-2. Recently the Vpu protein has been reported to have three biological functions: augmentation of virus particle release, degradation of newly synthesised CD4, and down regulation of the surface expression of MHC class I molecule (Kerkau et al., 1997).

iii. Genotypic and phylogenetic analysis of HIV-2

Sequences of HIV-2 viral proteins are only distantly related to that of HIV-1. Amino-acid sequence homology between HIV-2 and HIV-1 varies from ~ 40% in Env to ~ 60% in Pol and Gag (Guyader et al. 1987). HIV-2 is much closer to SIV_{mac} or SIV_{sm} as the amino-acid homology is 70-75% in Env and ~85% in Pol and Gag (Chakrabarti et al. 1987; Hirsch et al. 1989).

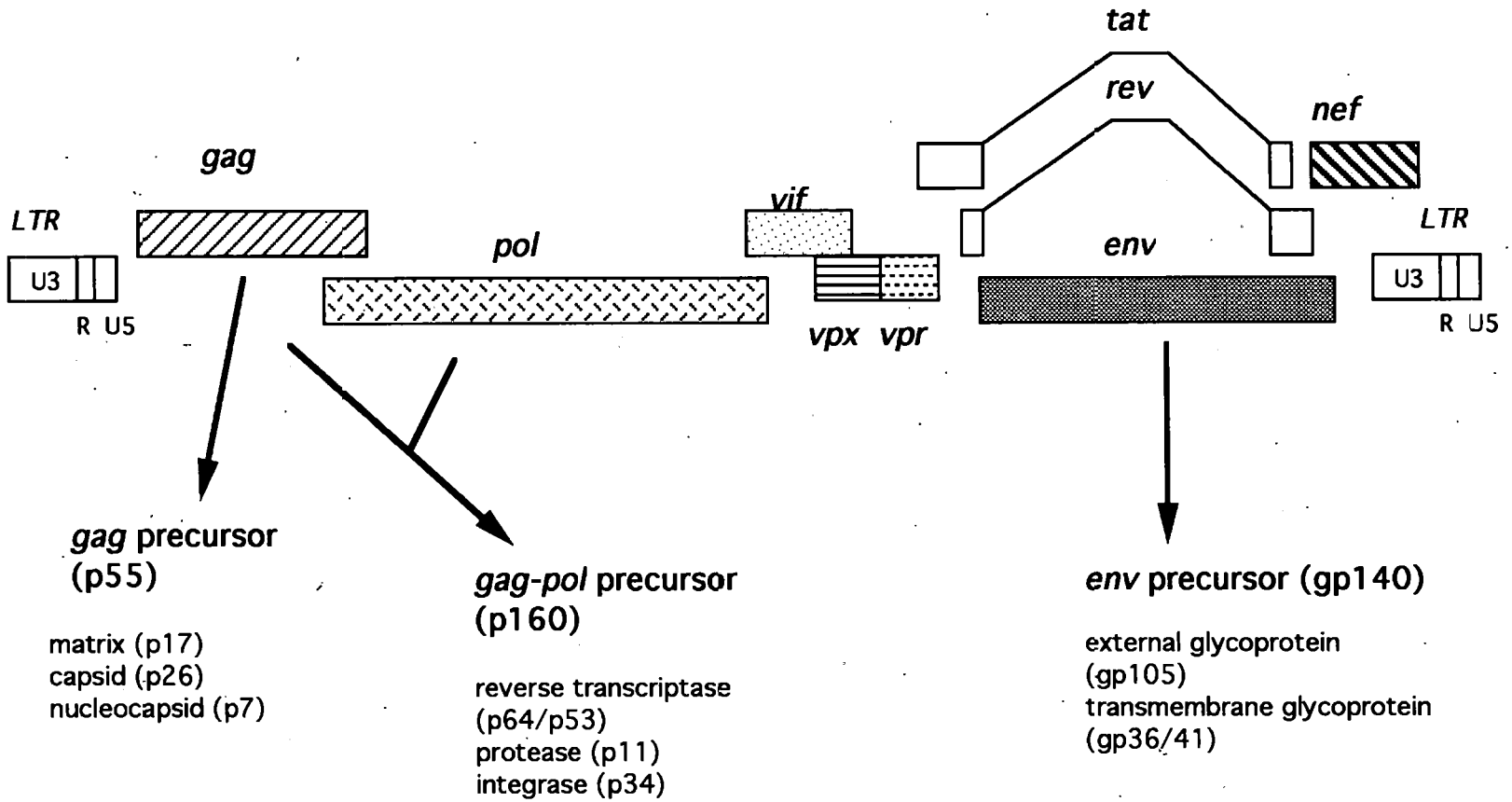

Figure 1.2. Genomic organisation of HIV-2 and the major virion-encoded proteins

A high degree of sequence diversity has also been noticed in HIV-2 viruses and it has been postulated that multiple independent introductions of genetically diverse SIV_{sm} viruses into human population occurred (Sharp et al., 1994). So far five subtypes (A-E) have been described but only subtype A and B which are associated with clinical disease, have been successfully isolated (reviewed by Sharp et al. 1994). There have been only single representatives of subtype C,D and E. The sequence of subtype D is so close to SIV_{sm} or SIV_{mac} viruses that the virus could not be separated into distinct phylogenetic lineages from viruses of primate origin (Gao et al. 1992). Geographical distribution of HIV-2 subtype has not been extensively studied yet. Limited studies have shown that sequences of HIV-2 *gag* gene in 67 HIV-2-infected individuals living in a rural village, Guinea-Bissau were all subtype A (Xiang et al, 1996). Non-A subtype has not been reported as a dominant subtype in any country.

Recombination of HIV-1 subtypes takes place (Robertson et al., 1995; Myers et al., 1995). Recombination of HIV-2 subtypes has also been reported. $HIV\text{-}2_{7312A}$ which originated from HIV-2 patient from Abidjan, Côte d'Ivoire, has a mosaic genome of HIV-2 subtype A and subtype B (Gao et al. 1992).

Information on intra-patient variability of HIV-2 has been limited but one small study with 5 HIV-2 infected patients suggested that the average intra-patient nucleotide variability rate was lower among healthy patients than among patients with AIDS (Sankale et al., 1995).

iv. Regulation of HIV-2 gene expression

a. Structure of HIV-2 LTR is different from that of HIV-1 LTR

The HIV long terminal repeat (LTR) contains *cis*-acting viral elements which play a crucial role in the regulation of HIV RNA synthesis and is divided into three functional distinct regions designated U3, R, and U5. Nucleotide sequence homology between HIV-1 and HIV-2 LTRs is low, 30-40% (Guyader et al., 1987). The U3 domain of both HIV-1 and HIV-2 LTRs contains basal promoter elements, including a TATAA box and SP1 binding sites. Additional *cis*-acting sequences in the U3 domain differ considerably between HIV-1 and HIV-2. The HIV-1 LTR has two κB regulatory elements whereas the HIV-2 LTR does not have a tandem repeat of the κB regulatory element in the region but contains other elements: PuB1, PuB2 and Pets which are not found in HIV-1 (Markovitz et al., 1990, 1992; Tong-Starksen et al., 1990) (Figure 1.3). Mutation experiments suggested that activation of the HIV-2 enhancer is dependent on at least the four *cis*-acting elements (Markovitz et al., 1992). Later, a novel *cis*-acting element has been identified immediately upstream of the κB site, designated peri-κB, in the HIV-2 LTR but not in the HIV-1 LTR (The peri-κB site is not shown in Figure 1.3). The factor(s) binding to the peri-κB site has not been identified but appears to be monocyte-specific (Clark et al., 1995).

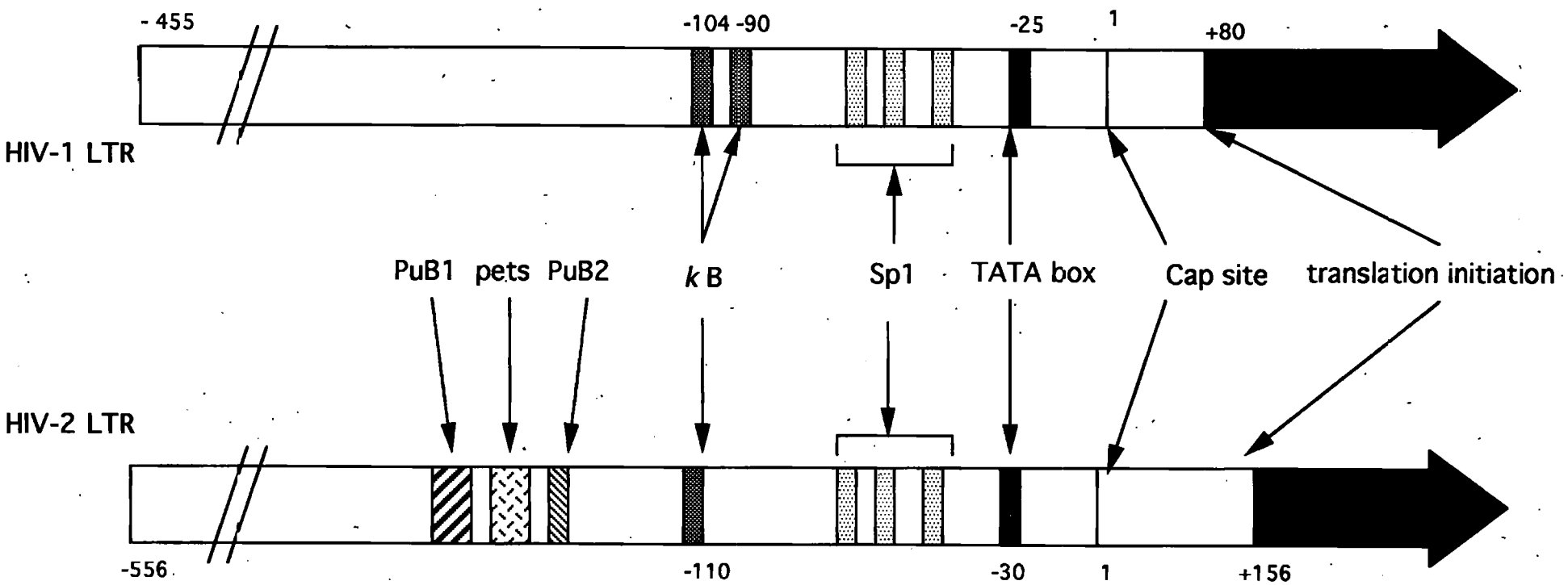

Figure 1.3. Comparison of the enhancers in the U3 domains of the HIV-1 and HIV-2 LTRs.
The *cis* -acting DNA elements located in the viral LTR are indicated as filled boxes; the figure was modified from a reference (Hannibal et al., 1993)

b. Conditions which activate the enhancer are different between HIV-1 and HIV-2

Not surprisingly the HIV-1 and HIV-2 enhancers are induced differently. For example, a cytokine, tumour necrosis factor alpha (TNF-α) induces HIV-1 transcription primarily through the two κB elements in the HIV-1 enhancer by inducing a DNA binding protein, NFκB (reviewed by Matsuyama et al., 1991). On the other hand, HIV-2 transcription is only weakly induced by TNF-α perhaps because NF-κB does not strongly activate the single κB element in the HIV-2 enhancer (Hannibal et al., 1993). Another example is that the triggering of the CD3 component of the T-cell receptor complex with anti-CD3 antibodies stimulates HIV-2 transcription but not HIV-1 (Markovitz et al., 1990). This stimulation of HIV-2 with anti-CD3 antibodies was thought to be mediated by a different cellular factor, AP3, binding to an upstream enhancer which is not present in the HIV-1 LTR (Tong-Starksen et al., 1990; Markovitz et al., 1992). Further understanding of the difference in transcriptional enhancers of HIV-1 and HIV-2 in wild-type HIV is warranted.

c. HIV-2 regulatory genes and their response elements in RNA

Regulation of HIV gene expression is also controlled by two viral regulatory proteins: Tat and Rev. Both Tat and Rev are nuclear proteins that bind to the viral RNA at specific sites called the Tat-responsive element (TAR) and the Rev-responsive element (RRE), respectively. Tat augments level of viral RNA at the transcriptional level whereas Rev acts at the post-transcriptional level and is essential for the expression of the viral structural proteins such as the *gag/pol* and *env* mRNA (reviewed by Felber & Pavlakis, 1993).

The structure of HIV-2 Tat is similar to that of HIV-1 Tat (Elangovan et al., 1992). However the secondary structure of HIV-2 TAR differs considerably from that of HIV-1 TAR as HIV-2 TAR can be folded into a complex structure with three stem-loops whereas HIV-1 TAR can be folded into a simple one-stem structure (Fenrick et al., 1989; Berkhout

1992). Interestingly HIV-2 Tat *trans*-activates the HIV-1 LTR less efficiently than HIV-1 Tat while both HIV-2 Tat and HIV-1 Tat efficiently *trans*-activate the HIV-2 LTR (Emerman et al., 1987). The amino-acid differences in the basic domains but not the core domains of the Tat proteins appear to be responsible for this incomplete reciprocity (Elangovan et al., 1992).

HIV-2 Rev and HIV-1 Rev are also functionally and structurally similar. However while HIV-1 Rev can efficiently complement an HIV-2 provirus lacking a functional HIV-2 Rev protein, HIV-2 Rev can not effectively substitute for HIV-1 Rev (Lewis et al., 1990; Malim et al., 1989; Sakai et al., 1991).

d. Significance of nef gene.

The importance of Nef in the AIDS pathogenesis has been demonstrated in the rhesus monkey model where functional *nef* expression is required for both high viral load and disease progression (Kestler et al., 1991). The importance of Nef was also suggested in HIV infection of humans as a *nef*-defective HIV-1 has been found in several long-term non-progressors (Deacon et al., 1995; Kirchhoff et al., 1995). Therefore it is crucial to understand the biological function of the *nef* gene. Several *in vitro* experiments have shown that the HIV-1 Nef protein has several biological activities: down regulation of cell surface expression of CD4 (Garcia et al., 1991; Aiken et al., 1994), augmentation of infectivity of HIV-1 particle (Chowers, et al., 1994; Miller et al., 1994; Spina et al., 1994), activation of infected cells (Du et al., 1995), down regulation of MHC class I molecule (Schwartz et al., 1996), and up-regulation of Fas Ligand expression (Xu et al., 1997). The last two activities may be responsible for the virus evading cytotoxic T-cell response. The function of down regulation of CD4 expression was thought to render cells resistant to viral superinfection (Benson et al., 1993). On the other hand very little study has been done on the function of

HIV-2 Nef obtained from the early stage of infection. Deletion of *nef*-defective wild-type HIV-2 has not been reported yet.

v. In vitro biological characteristics of HIV-2

Biological properties of HIV-2 are similar to HIV-1. The first prototype of HIV-2, HIV-2_{ROD} obtained from a West African with AIDS, grows rapidly to a high titre *in vitro* and shows cytopathic effects on cultured cells similar to that of HIV-1 such as syncytia-formation and cell lysis (Clavel et al. 1986). Less cytopathic HIV-2 strains, HIV-2_{UL1} from a patient with neurologic symptoms and HIV-2_{ST} from an asymptomatic individual, have been reported. They do not form syncytia, nor do they kill CD4-bearing cells or down-regulate cell surface CD4 (Evans et al., 1988; Kong et al., 1988). As has been well-described in HIV-1 (Asjö et al., 1986; Cheng-Mayer et al., 1988; Tersmette et al., 1988), diversity of biological properties exists among HIV-2 isolates and these correlate with clinical status. Isolates with high replicative ability which replicate in tumour cell lines and induce syncytia were obtained from symptomatic patients with a low $CD4^+$ cell count whereas less replicative isolates which lack the ability to replicate in tumour cell lines or to induce syncytia were obtained from asymptomatic individuals and from some symptomatic patients (Albert et al., 1990a; Schulz et al., 1990). Simon et al., reported that the rate of isolation was significantly lower in HIV-2 than HIV-1 among patients with a relatively high $CD4^+$ cell count (Simon et al., 1993). This finding may also reflect on the lower replication-capacity of HIV-2. However in this study the *in vitro* replication-capacity of HIV isolates was not studied. More recently biological properties of HIV-2 have been related to genotype of V3 loop; the V3 loop amino acid sequence was more heterogenous and had higher net charge in viruses with rapid/high replication capacity (Albert et al., 1996). A comparison of biological properties between HIV-1 and HIV-2 in isolates from community-based subjects yet to be made.

vi. Cell tropism of HIV-2

Like HIV-1, HIV-2 also uses the CD4 molecule as the primary cell attachment receptor (Dalgleish et al., 1984; Sattentau et al., 1988) and HIV-2 can infect a similar range of CD4 expressing human T-cell and macrophage cell lines as that of HIV-1 (Evans et al., 1988; Franchini et al., 1989; Albert et al., 1990a; Schulz et al., 1990). However some HIV-2 strains can also infect certain $CD4^-$ human cell lines and this infection is enhanced by soluble CD4 (Clapham et al., 1992).

Recent discoveries of chemokine receptors which act as second receptors for HIV-1 entry have provided more insights into mechanisms of the diversity of cell tropism (Feng et al., 1996; Deng et al., 1996; Dragic et al., 1996; Alkhatib et al., 1996). Limited information on chemokine receptor usage among HIV-2 viruses have suggested that HIV-2 laboratory established strains and primary isolates use the same chemokine receptors as HIV-1. CCR5 or CCR3 were used by HIV-2 isolates from asymptomatic individuals whereas CXCR4 was used by HIV-2 T-tropic strains and isolates from AIDS patients (Sol et al., 1997). However the pattern of co-receptor usage seems to be more complex and wider for HIV-2 than HIV-1 since an HIV-2 envelope protein has been shown to use a wider repertoire of chemokine receptors including CCR1, CCR2, CCR4, and CXCR2, to induce fusion in a cell-to-cell fusion assay (Bron et al., 1997) and also primary isolates of fast-growing HIV-2 have been shown to use multiple chemokine receptors: CCR1, CCR2b, CCR3, CCR5 and CXCR4 (Heredia et al., 1997; McKnight et al., manuscript submitted). The differences in the usage of chemokine co-receptor may be important for determining the difference in the pattern of disease. Further studies of co-receptor usage for primary isolates with slow/low growing capacity from HIV-2 long-term non-progressors are needed.

1.a.5. Host-immune response to HIV-2

i. Humoral immunity

Neutralising antibodies (NA) against HIV-1 viruses have been extensively investigated since early stage of HIV-1 research (Weiss et al., 1985, 1986; Robert-Guroff et al., 1985) and neutralisation targets on HIV-1 Env have been well-characterised: these are linear epitopes in the third (V3) and the second (V2) hyper-variable domains (Matsushita et al., 1988; Javaherian et al., 1989; Broliden et al., 1990; Fung et al., 1992; McKeating et al., 1993) of external glycoprotein, gp120, and also in transmembrane glycoprotein, gp41 (Muster et al., 1993). Non-linear, conformationally sensitive epitopes as well have been described in the CD4 binding region of gp120 (Lasky et al., 1987; Steimer et al., 1991, Kang et al., 1991; Thali et al., 1991). Fewer studies of NA in HIV-2 infection have been done. Like HIV-1, it has been suggested that there were neutralisation targets on the exposed domains of HIV-2 Env: in the V2 and the V3 loop and one conformational epitope outside V1, V2, and V3 (Bjorling et al., 1994; Traincard et al., 1994; McKnight et al., 1996), though more studies are needed as previous reports have shown contradictory results (Norrby et al., 1991; Babas et al., 1994; Matsushita et al., 1995). Emergence of NA escape viruses in HIV-1-infected individuals has been observed by several groups (Albert et al., 1990b; Homsy et al., 1990; Tremblay & Wainberg 1990; Montefiori et al., 1991). However study of anti-HIV-2 NA in clinical samples has been limited; one study showed that NA against autologous virus isolates have been readily demonstrated in 9 HIV-2-infected individuals (Bjorling et al., 1993), though the autologous NA titre was not related to the rate of HIV-2 disease progression. NA escape virus has not been described in HIV-2-infected individuals. NA in sera from HIV-2-infected individuals have been shown to cross-neutralise SIV (Robert-Guroff et al., 1992) and also HIV-1 laboratory strains (Weiss et al., 1988; Bottiger et al., 1990). Nevertheless, cross-neutralising against HIV-1 primary isolates have not been studied.

A few studies have shown the existence of antibody-dependent cellular cytotoxicity (ADCC) in sera from HIV-2-infected individuals (Björling et al., 1991; Von Gegerfelt et al., 1993) but the importance of ADCC remains largely unknown.

ii. Cell-mediated immunity

Cytotoxic T-cells (CTL) are thought to play an important role in eradicating virus in infected cells during acute infection and in controlling viral replication in persistent infection (reviewed by McMichael & Walker, 1994). Extensive studies of HIV-1-specific CTL have shown that in most HIV-1-infected asymptomatic individuals CTL can be demonstrated against the structural proteins Gag, Pol and Env (Walker et al., 1988; Nixon et al., 1988; Lamhamedi-Cherradi et al., 1992), that the precursor frequency of HIV-1-specific CTL is unusually high in such individuals (Hoffenbach et al., 1989; Gotch et al., 1990; Carmichael et al., 1993), and that high levels of CTL are associated with the slow rate of disease progression (Rinaldo et al., 1995a; Riviere et al., 1995; Klein et al., 1995). Albeit HIV-1 persists in most infected individuals, as the virus can acquire mutations to escape the CTL immune response and the emergence of such CTL escape mutants appears to associate with progression to AIDS (Phillips et a., 1991; Goulder et al., 1997). It has also been shown that the rapid reduction of primary plasma viraemia coincided with the development of HIV-1-specific CTL (Koup et al., 1994; Borrow et al., 1994) but not the neutralising antibodies against autologous viruses (Ariyoshi et al., 1992; Connick et al., 1996). More recently a rapid selection of CTL escape viruses during primary infection has been demonstrated (Borrow et al., 1997) and the induction of memory CTL, particularly those against Env, during early infection has been related with slower rate of disease progression (Musey et al., 1997).

Studies of CTL activities in HIV-2 infection have been limited. CTL activities in HIV-2-

infected individuals were first studied by Gotch who showed that HIV-2 Gag CTL were demonstrable in fresh PBMC in 5/7 (70%) of HIV-2-infected individuals and identified a B53 restricted CTL epitope in HIV-2 Gag (Gotch et al., 1993). CTL activities in HIV-2-infected individuals have also been studied in Chapter 5 of this thesis where it has been demonstrated that HIV-2-specific CTL, predominantly targeting Gag, were detected in all HIV-2-infected asymptomatic individuals. This CTL activity was inversely correlated with HIV-2 proviral load (Ariyoshi et al., 1995; Chapter 5). Later, additional CTL epitopes, restricted by HLA B35 and B5801, were found in HIV-2 Gag, Pol and Nef. Of those epitopes, amino acid sequence in a Nef epitope, restricted by B35 was completely conserved between HIV-1 and HIV-2. Interestingly although there are some differences in amino acid sequences between HIV-2 Gag or Pol CTL epitopes restricted by B35 or B5801 and the corresponding regions in HIV-1 Gag or Pol, the corresponding HIV-1 peptides were cross-reactively recognised by HIV-2 CTL (Rowland-Jones et al., 1995; Bertoletti, et al., 1998). CTL escape mutant from HIV-2-specific CTL has not been described yet.

1.b. Review of serodiagnosis of HIV-infections in West Africa

The serodiagnosis of HIV-infection is complex in West Africa because of the presence of both HIV-1 and HIV-2. Especially the significance of dual seroreactivity, which is frequently observed in the region, remains unclear as both HIV-1 and HIV-2 proviruses were detected only in about half of dually seroreactive individuals (George et al., 1992; Peeters et al., 1994). This issue is further discussed in Chapter 7. Accurate serological diagnosis of single HIV-1 or HIV-2 infections and of HIV-1/HIV-2 dual infection is essential for seroepidemiological studies, for the understanding of the pathogenic properties of the two viruses, and for the study of interaction between the two types of HIV.

Because of significant amino acid sequence homology between HIV-1 and HIV-2, cross-

reactive antibodies are common. The WHO recommended criterion for HIV-2 positivity on Western blot depends on the antibody recognition of envelope bands (World Health Organisation 1990). However even the Env protein which is known to be less homologous than Gag or Pol protein, is often recognised by heterotypic sera in the Western-Blot assay; cross-reactivity seems to be more extensive for HIV-2 sera against HIV-1 antigens than the reverse (Tedder et al, 1988; De Cock et al, 1991a). To increase the specificity of HIV antibody assays, synthetic peptides corresponding to the immunodominant region of transmembrane glycoprotein gp36/gp41 of HIV-1 and HIV-2 where the antibody responses are type-specific, have been used (Norrby et al., 1987; Gnann et al. 1987; Baillou et al, 1991). These peptide-based methods were found to be more specific than Western-Blot (De Cock et al, 1990, 1991b). Type-specific competitive ELISA assays using recombinant Env protein were also shown to be highly specific (Tedder et al., 1988) and by titrating antibody with the competitive ELISA assays, it is possible to distinguish true dual seroreactivity from cross-reactivity (Berry et al, 1993). The latter technique has been further pursued in a large number of subjects in Chapter 7.

1.c. The role of HIV-1 viral load in HIV-1 pathogenesis

1.c.1. HIV-1 pathogenesis.

Although it has been convincingly shown that HIV infection leads to AIDS (Darby et al., 1995), the real mechanisms of immunosuppression caused by HIV-1 have not been clearly understood. A number of hypotheses have been raised (reviewed by Weiss, 1993; Pantaleo, 1993b). These are a) direct killing of HIV-infected cells by HIV-mediated cytopathic effects, b) killing of HIV-infected cells by HIV-specific immune responses, c) killing of HIV-uninfected bystander cells by cell-to-cell fusion, d) autoimmune destruction triggered by the virus through molecular mimicry of cellular antigens, e) apoptosis induced by HIV viral gene products for instance HIV-1 Tat, Env (Westendrop et al., 1995; Li et al., 1995),

e) dysfunction of antigen-presenting cell inducing an imbalance in the T_H1-type and T_H2 type responses (Meyaard et al., 1993; Clerici & Shearer, 1993). This thesis focuses on the role of HIV viral load in the pathogenesis, which does not necessarily exclude other mechanisms of pathogenesis. The role of HIV viral load is discussed in detail below. The Possible role of CTL in the immuno-pathogenesis is also discussed in Chapter 5 in the context of HIV-2 infection.

1.c.2 HIV-1 virus population dynamics *in vivo*

The virus has two phases in its life cycle: RNA virus as a virion existing outside cells and provirus which is DNA copy of viral RNA existing inside cells. Thus both RNA and DNA viral load need to be measured. However in the early days of the studies of HIV-1 viral load, only the technique to quantify DNA viral load was available and the importance of the virus in lowering $CD4^+$ cell count was controversial, because during the asymptomatic phase the level of HIV-1-infected $CD4^+$ cells was thought to be too low to cause progressive $CD4^+$ cell loss (Sheppard et al., 1993). Since the advent of very potent anti-retroviral drugs and of accurate assays to quantify HIV-1 RNA virus in plasma, two groups have successfully demonstrated that by carefully measuring the decay of HIV-1 virus in plasma in patients receiving potent anti-retroviral drug that not only the virus but also productively infected cells turn-over very rapidly with a short half life of few days (Wei et al., 1995; Ho et al., 1995). This insight into the dynamics of the HIV-1 virus and virus-infected cells has at least partly answered the question as to why patients lose $CD4^+$ cells despite a low frequency of HIV-infected cells. Later more detailed studies on HIV-1 RNA viral kinetics and HIV-1 provirus have been published (Perelson et al., 1996, 1997; Chun et al., 1997). The current view of HIV-1 population dynamics *in vivo* is summarised in a schematic diagram (Figure 1.4). In five HIV-1-infected patients with mean plasma virions of 216,000 (range, 12,000, 643,000) copies per ml, the half life of HIV-1 RNA in plasma was

found to be very short, approximately 6 hours and on average $10x10^9$ virions were estimated to be produced every day (Perelson et al., 1996). Thus a substantial number of cells must be actively producing HIV-1 viruses to maintain this level of viral RNA in plasma. The observed exponential decline of HIV-1 RNA in plasma in the patients on anti-retroviral drug therapy implies that the half life of such productively infected cells is also very short, about 1.6 days (Perelson et al., 1996), though there is small proportion (<1%) of cells which are long-lived despite being productively infected; the half life of these cells being 1-4 weeks (Perelson et al., 1997). Therefore as the diagram shows, the major contributor to cell death by HIV is the productive infection of cells where the number of productively infected cells is reflected by the level of viral RNA in plasma.

The majority of productively infected cells are thought to result from *de novo* infection of $CD4^+$ cells. Whether a newly infected cell becomes latently infected or productively infected, appears to depend upon the state of activation of the cell: if the cell is activated when infected with HIV-1, productive infection results whereas if the cell is resting, the infection becomes latent (Stevenson et al., 1990). Therefore the size of the population of activated cells susceptible to HIV-1 infection determines the number of productively infected cells. Chun et al., showed that the vast majority of HIV-1 proviruses are in either unintegrated form or integrated form but replication-incompetent, though there are some resting T-cells which are latently infected with replication-competent HIV-1 proviruses which has integrated. However the total load of such cells is estimated to be low ($\leq 10^7$ cells) (Chun et al., 1997). Thus although these latently infected resting T-cells can be reactivated to produce the virus, the number of viruses produced by such cells appears to be small (Wei et al., 1995; Ho et al., 1995).

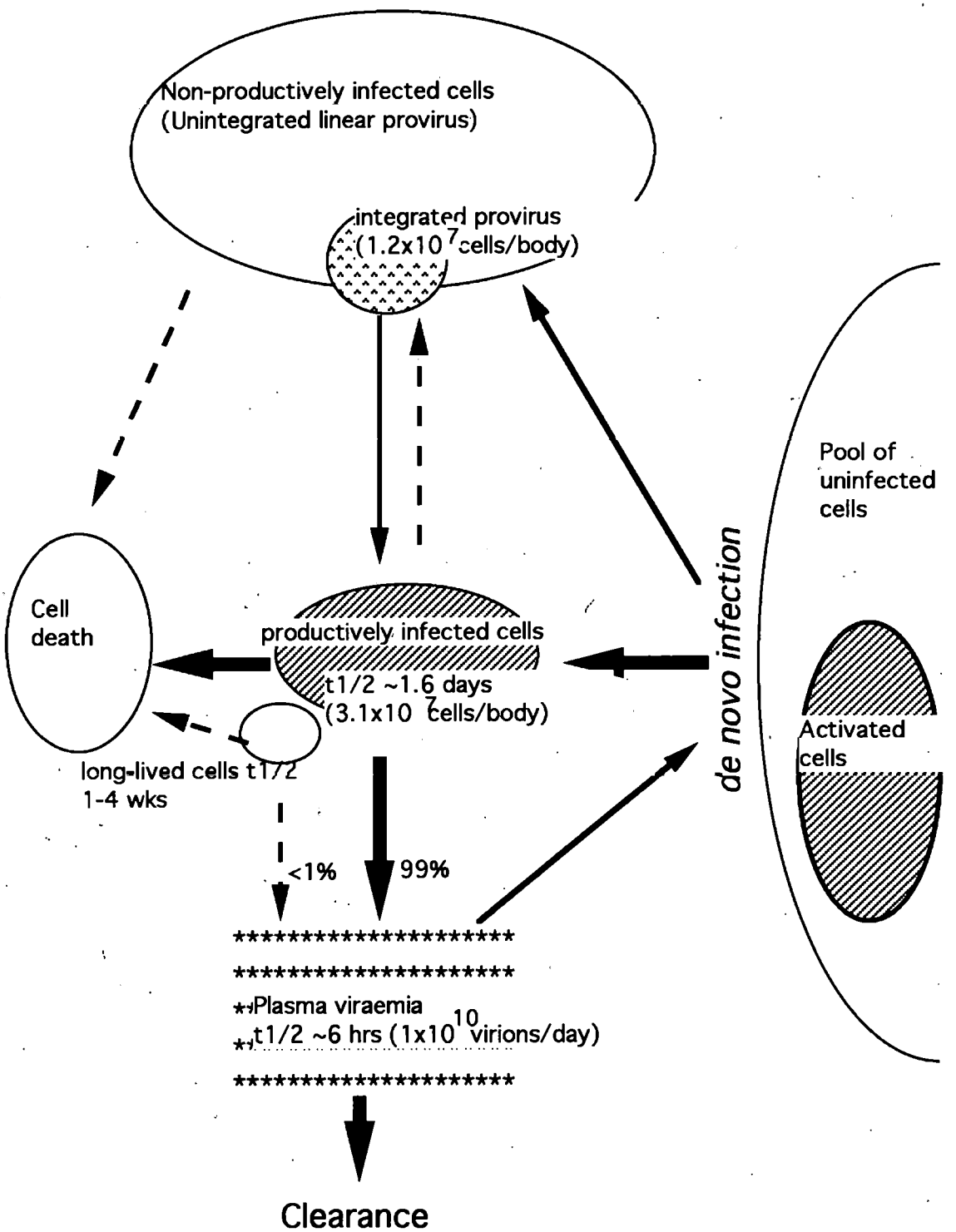

Figure 1.4. Schematic diagram of HIV-1 population dynamics in vivo. The figure shows estimated half life ($t_{1/2}$) (Perelson et al., 1996 & 1997) and estimated numbers of virions produced per day (Perelson et al., 1996) and cells (Chun et al., 1997).

1.c.3. Measurement of HIV-1 viral load

i. Viral load in peripheral blood versus lymphoid tissue

Because of its accessibility most investigators have used peripheral blood to measure viral load on the assumption that the level of virus in plasma and in peripheral blood mononuclear cells reflects the total amount of RNA virus or provirus in the body. However the major site for viral replication appears to be in lymphoid tissue (Armstrong & Horne 1984; Pantaleo et al., 1991, 1993a; Embretson et al., 1993). A study of HIV-1 and HIV-2 infection in The Gambia has recently shown that although the frequency of infected cells was, on average, higher in lymph-node than in peripheral blood, the values at the two sites were closely correlated (Jobe et al., manuscript in preparation). Moreover there is vigorous lymphocyte circulation between lymphoid tissue and peripheral blood, the mean transit time of a lymphocyte in peripheral blood being only 30 minutes, and roughly $500x10^9$ lymphocytes travel through the blood each day, which is about the same as the number of lymphocytes within the whole body (Westermann & Pabst 1990). Thus measurement of viral load in PBMC is likely to be representative of the total population of lymphocytes in the body.

HIV-1 virions produced in lymphoid tissue are assumed to diffuse into lymphoid fluid and eventually enter into the venous blood. Some HIV virions are trapped by follicular dendritic cells in lymph-nodes (Armstrong & Horne 1984; Pantaleo et al., 1993a; Embretson et al., 1993). However the quantity of HIV-virions trapped by these cells in the lymph-node has not been accurately estimated but appears to be negligible as compared with total number of virions produced into plasma. Two reports have suggested that there is a positive correlation between plasma viraemia and proviral DNA load in the lymphoid tissue (Sei et al., 1994; Dianzaniet al., 1996).

ii. Methods used to measure viral load

Different techniques have been applied to evaluate viral load. The early HIV-1 viral load studies were done using limiting dilution tissue-culture techniques to determine tissue-culture-infective doses (Ho et al., 1989; Coombs et al., 1989) so was a HIV-2 viral load study (Simon et al., 1993). The tissue-culture method has the advantage that it can quantify replication competent viruses but the method is tedious and also is not sensitive especially when detecting viruses in plasma of patients with a high $CD4^+$ cell count (Coombs et al., 1989; Venet et al., 1991; Saag et al., 1991; Dewar et al., 1992; Simon et al., 1993; Ariyoshi et al., 1994). Moreover results are often difficult to interpret because of variation in the susceptibility of donor cells (Wainberg et al., 1987; Cloyd & Moore, 1990; Paxton et al., 1996) and in HIV-2 infection, the detection of the virus in the culture may be often limited by the slow/low replication property of HIV-2.

PCR techniques were applied first to quantify viral load using the limiting dilution technique (Schnittman et al., 1989; Simmonds et al., 1990; Zhang et al., 1991; Hsia & Spector, 1991) and later without this step. Most techniques use external controls to estimate number of viral DNA copies (Genesca et al., 1990; Lee et al., 1991; Yerly et al., 1992; Ferre et al., 1992; Escaich et al., 1992; Bieniasz et al., 1993; Chevret et al., 1994) or viral RNA copies (Holodniy et al., 1991; Aoki-Sei et al., 1992, Semple et al., 1993). The number of HIV viral RNA was not sufficiently high to be detected by direct extraction of RNA using the guanidinium thiocyanate-phenol-chloroform extraction method or polyethylene glycol method (Holodniy et al., 1991; Semple et al., 1991). Early investigators therefore used ultra-centrifugation (Zhang et al., 1991; Ottmann et al., 1991; Aoki-Sei et al., 1992; Piatak et al., 1993) or immuno-precipitation (Semple et al., 1993) to concentrate the virions in plasma in order to improve the sensitivity. In this thesis and also in other studies a technique described by Boom et al., was used in which viral RNA was precipitated by silica particles

(Boom et al., 1990; van Gemen et al., 1993, 1994; Berry et al., submitted). This RNA extraction technique is also incorporated into a commercial assay, NASBA. Another technique to measure viral RNA in plasma is branched-DNA which relies on signal amplification rather than the PCR amplification. Nevertheless the technique is less sensitive than PCR-based methods and it is difficult to develop as home-made assays.

The use of external controls may result in falsely low measurements due to the presence of non-specific inhibitors of PCR in blood samples, such as residual heparin or haem containing proteins, causing tube-to-tube variation of efficiency of PCR amplification. To over-come this problem, competitive PCR techniques have been developed, in which internal controls have been spiked into the clinical samples (Scadden et al., 1992; Piatak et al., 1993; van Gemen et al., 1993). However, the use of adequate RNA purification methods, such as the method of Boom, usually overcomes problems of non-specific inhibitors and allows the use of external controls which are less time consuming.

Recently several commercial assays have become available to quantify HIV-1 RNA viral load: these are a PCR-based assay with internal controls (Amplicor, Roch), an RNA amplification method with internal controls (NASBA, Organon), and a branched DNA assay with external controls (BDNA, Chiron). However as most HIV-1 subtypes in The Gambia are not subtype B viruses (Ariyoshi, et al., 1996), the sensitivity of those commercial assays for detecting non-type B viruses may not be as high as that for type B viruses (Loussert-Ajaka, et al., 1995; Clewley et al., 1995). Moreover no commercial or non-commercial molecular based assay for measuring HIV-2 DNA or RNA has been available until recently (Berry et al., 1994, manuscript submitted).

1.c.4. HIV-1 viral load as a marker of disease progression

Although all early studies have shown that HIV-1 proviral load is inversely correlated with patient's $CD4^+$ count or with their disease status (Ho et al., 1989; Coombs et al., 1989; Simmonds et al., 1990; Holodniy et al., 1990; Lee et al., 1991; Aoki-Sei et al., 1992; Yerly et al., 1992; Ferre et al., 1992; Bieniasz et al., 1993; Chevret et al., 1994), these cross-sectional observations did not indicate whether the increased viral load was the cause or the result of immunosuppression. Very few studies have shown that high proviral DNA load predicts the rate of disease progression (Schnittman et al., 1990). However the expression of HIV-1 mRNA in PBMC was shown to predict future HIV disease progression (Saksela et al., 1995). Furthermore since techniques to measure viral RNA load in plasma have become widely available and been applied to cohort studies, evidence has accumulated that RNA viral load is a strong prognostic marker of HIV-1 disease progression. Studies of HIV-1 seroconverters have shown the pattern of RNA viraemia after initial infection is predictive of clinical outcome (Mellors et al., 1995; Henrard et al., 1995; de Wolf et al., 1997) and a community-based cohort of homosexual men has shown that plasma viral load at entry to the study was a better predictor of progression to AIDS and death than was the $CD4^+$ cell count (Mellors et al., 1996).

1.c.5. HIV-1 long-term non-progressors

Although disease in HIV-1-infected individuals progresses faster than in HIV-2-infected individuals, a subgroup (approximately 5%) of HIV-1-infected individuals remain clinically healthy and immunologically normal for more than a decade and these individuals are termed long-term non-progressors (reviewed by Schrager et al., 1994). Several immunological and virological studies have been carried out to characterise long-term non-progressors (Cao et al., 1995; Pantaleo et al., 1995; Rinaldo et al., 1995a; Montefiori et al., 1996; Ferbas et al., 1995; Pilgrim et al., 1996). Most of these studies have consistently

shown low levels of viral load in long-term non-progressors and a combination of strong virus-specific immunity including cytotoxic T-lymphocyte activities and neutralising-antibody response. The better clinical outcome of some long-term non-progressors has been thought to be due to a viral genetic factor, a defective *nef* gene (Deacon et al., 1995; Kirchhoff et al., 1995) but the defect in the *nef* gene does not seem to be common in other long-term non-progressors (Huang et al., 1995; Michael et al., 1995).

More recently several groups have demonstrated that a host genetic factor, a 32 bp deletion in the CCR5 gene is associated with slow progression especially in early stage of infection (Dean et al, 1996; Michael et al., 1997a; Meyer et al., 1997; Stewart et al., 1997). Although another host genetic factor, a substitution of the valine at position 64 to isoleucine in the CCR2 gene has also been suggested to confer some protection against disease progression (Smith et al., 1997), the finding has not been repeated by other group (Michael et al., 1997b). Chemokines, MIP-1α, MIP-1β, and RANTES have been shown to inhibit HIV-1 and HIV-2 infection through blocking the interaction with the viral co-receptors (Cocchi et al., 1995). However neither serum levels of MIP-1α, MIP-1β and RANTES nor the production of these chemokines by in vitro stimulated cultured cells seems to correlate with disease progression (McKenzie et al., 1996; Clerici et al., 1996).

1.d. The aim of this thesis:

The AIDS unit at MRC Laboratories, The Gambia has addressed the general question, "Why do HIV-2-infected individuals live longer than HIV-1-infected individuals?" Answers to this question may enhance understanding of HIV-pathogenesis in general and also may provide a clue to prolong the life of HIV-1-infected individuals. As HIV-1 viral load plays an important role in HIV-1 pathogenesis, it is postulated that HIV-2 viral load also plays a

crucial role in HIV-2 pathogenesis. However studies of HIV-2 viral load have been very limited (Simmons et al., 1993, Berry et al., 1994). Thus the studies of HIV-2 viral load and the clarification of the *in vivo* HIV-2 replication in HIV-2-infected individuals at different clinical stages may provide important answers to the general question. For this reason, the role of HIV-2 viral load in HIV-2 pathogenesis has been the focus of this thesis which has been set up with the following aims:

1) To correlate HIV-2 viral load with the disease progression as determined by mortality or loss of $CD4^+$ cells.

2) To correlate HIV-2 viral load with HIV-2-specific cytotoxic T-lymphocyte activities.

3) To study the impact of co-infections such as HTLV-I and malaria on HIV-2 viral load.

4) To study the interaction between HIV-1 and HIV-2.

Chapter 2.

Materials and Methods.

2.a. Patients and materials

2.a.1. A community-based study in a rural village in Guinea-Bissau

The study village, Caio is located in the north-west of Guinea-Bissau where approximately 5000 adult people are living in a thick palm-tree forest (Figure 2.1). The main economic activities of the area are rice growing, palm oil and palm wine production. It is known that many men leave to work elsewhere in Guinea-Bissau, or in neighbouring countries in West Africa or in Europe. It is also known that many women leave to work and a significant proportion of those are involved in prostitution in the region's urban centres. Between August 1989 and May 1991, the first serosurvey was conducted (Wilkins et al. 1993). A total of 2770 subjects aged ≥15 years were tested and 220 subjects with HIV-2 infection were identified. Between March and June 1991, 132 HIV-2 seropositive subjects and 160 age and sex matched HIV seronegative controls were examined by a study clinician, and 10 ml of heparinised blood (preservative free heparin 10 IU/ml) was collected for CD4/CD8 subset analysis and for plasma and peripheral mononuclear cells (PBMC) (Ricard et al. 1994). Plasma samples were snap-frozen in liquid nitrogen and PBMC samples were cryopreserved with 10% dimethyl sulphoxide (DMSO) using liquid nitrogen vapour phase at the field laboratory. The population in the study village has been followed with annual censuses. These samples collected in 1991 were used in Chapter 3 and Chapter 6.a. In 1996, 86 HIV-2 seropositive subjects and 117 HIV seronegative controls who were recruited for the 1991 case-control study, were clinically and immunologically re-examined and their plasma and PBMC samples were stored as described above. In order to study the effect of malaria infection during the rainy season, 33 HIV seropositive and 22 HIV seronegative subjects were bled both in May and in October 1997. These samples were analysed in Chapter 6.b.

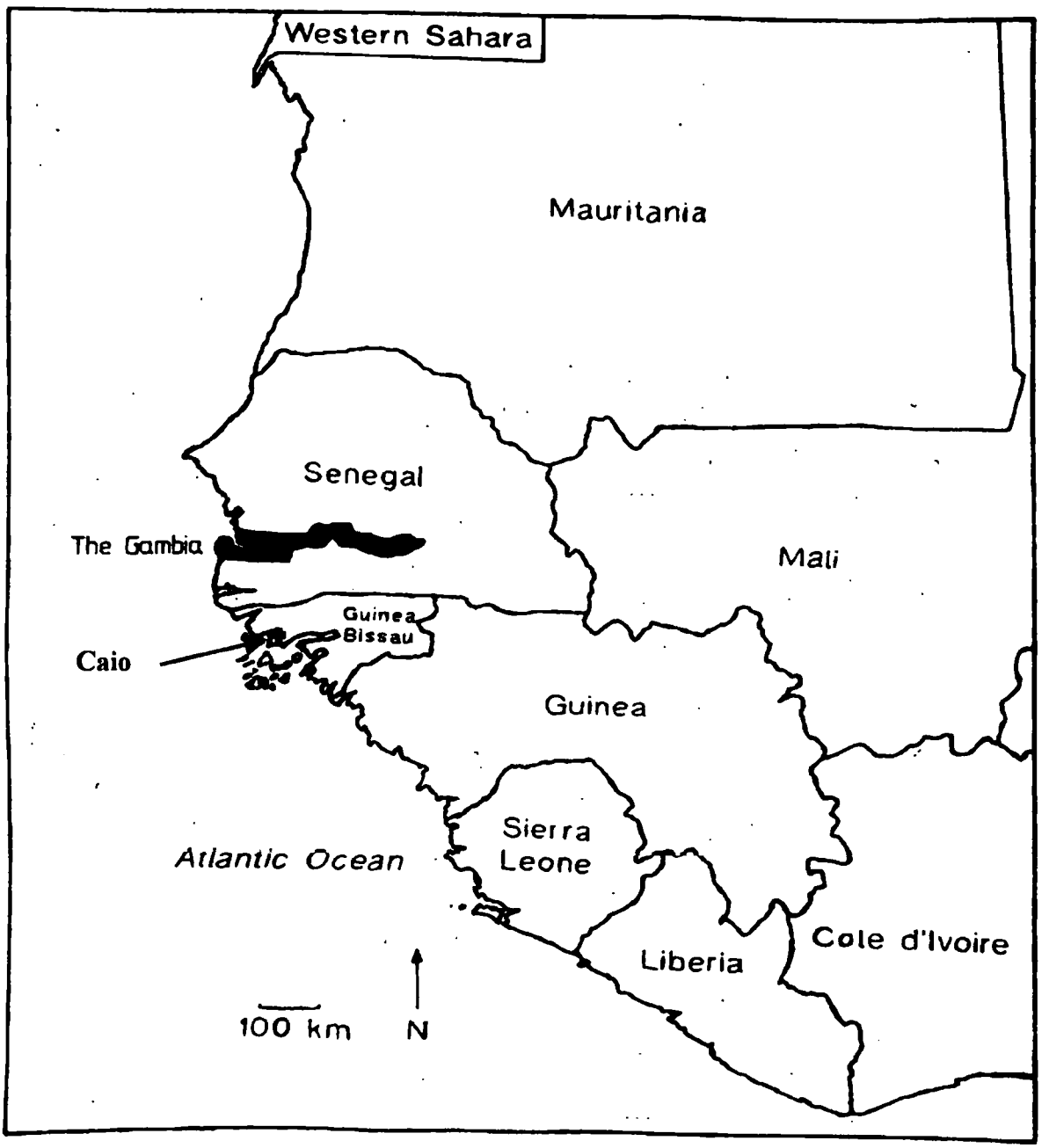

Figure 2.1. Map of West Africa.
The study village, Caio is indicated.

2.a.2. A hospital-based study at the MRC hospital in The Gambia

In The Gambia, which has a population of approximately one million, there are three major hospitals of which two, the Royal Victoria Hospital and the MRC hospital are located in the Western division where approximately half of the population lives (Figure 2.2). These hospitals serve as the referral centres for the entire country, and are open to all patients at minimal cost. HIV-testing is offered free of charge to patients with clinical symptoms suggestive of AIDS. Patients with TB, individuals with a high risk of infection such as commercial sex workers (CSWs), STD patients or spouses of HIV positive partners and all blood donors are also tested free of charge. Since May 1986 all patients who were diagnosed as HIV-seropositive at the two hospitals have been enrolled in a hospital-based cohort study. In addition, HIV seropositive CSWs who were identified by three cross-sectional serosurveys in 1988, 1989 and 1992, have also been enrolled. Patients were asked to attend at the MRC hospital to see the study clinicians every three months. Every six months, 15-20ml of heparinised blood was taken. PBMC were separated on Ficoll hypaque (Lymphoprep, Nycomed Pharma AS. Oslo, Norway), and then cryopreserved in liquid nitrogen; 1-3ml EDTA treated blood was taken to determine CD4/CD8 subset by FACScan (Beckton-Dickinson, Belgium) analysis. Study subjects in Chapter 4, 5, and 7 were recruited from this study.

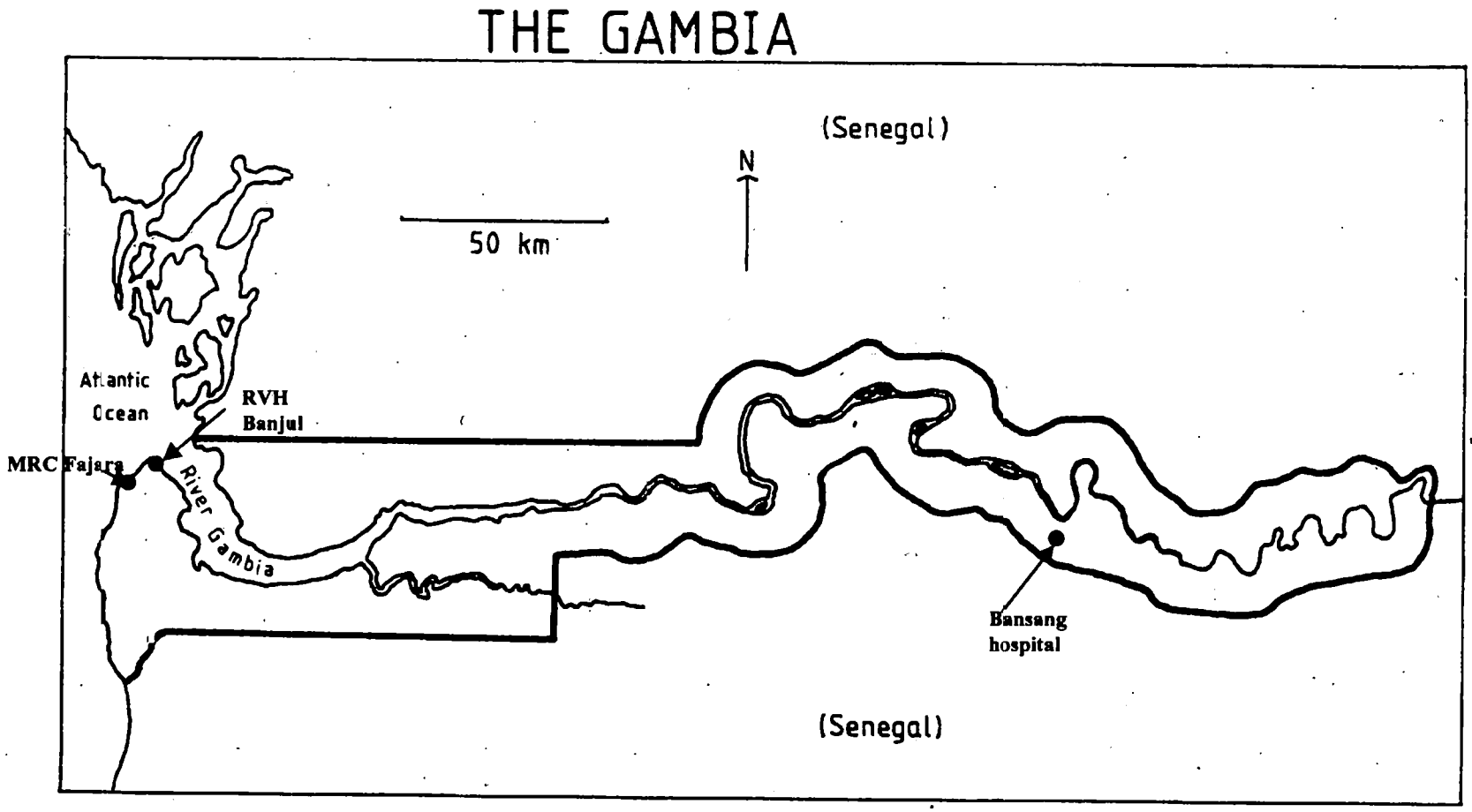

Figure 2.2. Map of The Gambia.
Three major hospitals are indicated.

2.a.3. A community-based perinatal transmission study in The Gambia

Between January 1993 and March 1995, 29,670 women attending the eight major government antenatal clinics were screened by serology for HIV (O'Donovan et al. 1996). This survey identified 110 HIV-1, 250 HIV-2 and 10 dually scropositive pregnant women. All women who were dually seroreactive were also enrolled in Chapter 7.

Verbal informed consent was obtained from all patients who were pre- and post-test counselled. All studies in this thesis were approved by The Gambia Government/MRC Ethical Committee.

2.b. Serological diagnosis.

2.b.1. Serological diagnosis of HIV infection.

HIV serostatus was initially screened by a combined competitive enzyme-linked immunosorbent assay (ELISA) (Combined Wellcozyme, Murex, U.K.). This screening assay is based on highly purified antigens of the core and envelope recombinant proteins of HIV-1 isolate (Sattentau et al., 1986). As the cross-reaction at the core protein alone is not satisfactory to detect all HIV-2 sera (Denis et al., 1988), an immunodominant epitope of the HIV-2 transmembrane envelope (Norrby et al., 1987), which was prepared by peptide sythesis techniques, was added to ensure sensitivity. If the sample was positive by the screening test, the type of HIV was determined by a type specific competitive ELISA (Wellcozyme I and Wellcozyme II, Murex, U.K.). The HIV-1-type specific ELISA is based on a fusion protein of p24-transmembrane envelope protein from HIV-1 isolate (Sattentau et al., 1986) and HIV-2-type specific competitive ELISA is based on transmembrane envelope protein from HIV-2 virus (Berry et al., 1993). If the sample was greater than the cut-off which was 3 standard deviations higher than the mean of the negative values for competitive ELISA, it was regarded as positive. If a sample was

positive for both HIV-1 and HIV-2 by the competitive ELISA assays, the type of HIV was determined by a peptide-based strip method (PeptiLAV, Diagnostics Pasteur, Marnes-La-Coquette, France). This technique is based on an immunoenzymatic strip method using two HIV-specific synthetic peptides which correspond to a type-specific immunodominant epitope of the transmembrane envelope glycoproteins :gp41 for HIV-1 and gp36 for peptide 2. These two peptides are fixed separately on the membrane. If the sample was + or ++ for both HIV-1 and HIV-2 bands in PeptiLAV, it was diagnosed as dually seroreactive.

2.b.2. Titration of anti-HIV-1 and anti-HIV-2 antibodies.

In Chapter 7, anti-HIV-1 and anti-HIV-2 antibody titres were investigated in dually seroreactive patients with or without PCR-proven HIV-1 and HIV-2 mixed infection. To determine titres of anti-HIV-1 and anti-HIV-2 antibodies, sera were serially diluted in ten-fold steps in normal human sera from 1:10 to 1:1000 before testing them with the type-specific competitive ELISA assays. The reciprocal value of the highest dilution at which ELISA was positive has been taken as titre of antibody.

2.c. PCR-based diagnosis.

The qualitative and quantitative PCR protocol for both HIV proviral DNA and RNA which is described here, was developed by Dr N Berry at University College London Medical School (Berry et al., 1994 & the manuscript submitted).

2.c.1. Extraction and quantification of cellular DNA.

DNA was extracted from cryopreserved PBMC obtained from heparinised blood using a previously described method (Berry et al., 1994). Sample PBMCs were thawed and washed once in 1 ml of PBS before being added to PCR-compatible lysis buffer {50mM KCl, 10mM Tris-HCl [pH 8.3], 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.4% Nonidet P-40,

0.45% Tween 20} with proteinase K (200 μg/ml). The volume of lysis buffer was determined to give a final concentration of $1x10^5$ cells per 10μl of lysis buffer, according to the number of PBMCs per vial which was counted by a counting chamber before cryopreservation. Lysed cells were incubated at 65°C for 2 hours in a water bath and vortexed before another 1 hour of incubation. Residual proteinase K was then inactivated by heating the cell lysate at >85°C for 15 minutes. The concentration of DNA in the extract was measured by a DNA fluorometer (TK 100 portable fluorometer, Hoefer Scientific Instruments, San Francisco USA) with fluorescent staining (Hoechst 33258 dye). The concentration was adjusted to a final concentration of 60μg/ml. All DNA samples were stored in a deep freezer at -70°C until its use.

2.c.2. Qualitative PCR for diagnosis of HIV infection.

PCR was conducted using 10μl (0.6μg) of DNA extract which is equivalent to 1×10^5 cells in a primary PCR tube in a total reaction volume of 50μl. For all diagnostic PCR, commercialy available PCR buffer {10mM Tris-HCl [pH 8.3], 50mM KCl} (GeneAmp®, Roche Molecular Systems, Inc, Branchburg, New Jersey, USA) was used with 1.5mM $MgCl_2$, a 200μM concentration of each 2'-deoxynucleoside 5'-triphosphate (dATP, dGTP, dTTP, and dCTP) (Pharmacia Biotech), 1 unit of *Taq* DNA polymerase (*AmpliTaq*®, DNA polymerase, Roche Molecular Systems, Inc, Branchoburg, New Jersey, USA) and 10pmol of each outer primer. For nested PCR, 2μl of the primary PCR product was transferred into a secondary PCR tube in a total reaction volume of 25μl which contains fresh PCR reagents and the inner primer set. Outer and inner primer sets which bind HIV-type specific but conserved regions in the LTRs were designed and provided by Dr N Berry. The sequence information is summarised in Table 2.1.

Hybaid thermocyclers (OmniGene thermal cycler, Hybaid limited, Middlesex) were used for all experiments. Thermocycling conditions for primary PCR was 94°C/4 minutes for denaturation (1 cycle), 94°C/1 minute of denaturation, 55°C/1 minute of annealing, 72°C/1 minute of extension (35 cycles), and 72°C/7 minutes final extension. Thermocycling conditions for secondary PCR was 94°/1 minute of denaturation, 50°C/1 minute of annealing, 72°C/1 minute of extension (25 cycles) and 72°C/7 minute of final extension. PCR conditions for HIV-1 and HIV-2 detection were the same except for the primer sets.

Secondary PCR products were run on a 1.5% agarose gel and visualised with ethidium bromide. If a single clear band was seen around the expected PCR product size: 141 bp for HIV-2 LTR, 120 bp for HIV-1 LTR, it was regarded as positive.

To avoid carry-over contamination for PCR diagnosis, the pre-PCR room, equipment, gowns, and gloves used for setting the PCR mixture were separated from the DNA extraction room, equipment, gowns and gloves used for extracting DNA, and from the post-PCR room, equipment, gowns, gloves used for handling PCR products as is recommended (Kwok and Higuchi 1989). Positive displacement pipettes with disposable capillaries and pistons (Microman®, Gilson Medical Electronics, France) were used to load the sample DNA.

2.c.3. Qualitative PCR for diagnosis of HTLV-I

PCR conditions for the detection of Human T-lymphotropic virus type I (HTLV-I) provirus were the same as that for detection of HIV, except for the annealing temperature which was 50°C and the primer sets which specifically bind HTLV *tax/rex* region (Table 2.1) (Tuke et al., 1992). Since the HTLV primers amplify the *tax/rex* region of both HTLV type-I and type-II (HTLV-II), differential diagnosis of HTLV-I and HTVL-II was made by

restriction enzyme analysis of the PCR product (Tuke et al., 1992) as follows: 5 µl of secondary PCR products were digested in a 20µl of reaction mix, containing 10 units of the restriction enzyme: *Sau*3A or *Taq*1, and 2µl of appropriate 10x reaction buffer. *Sau*3A digests were incubated for 90 minutes at 37°C and *Taq*1 digests were incubated for 90 minutes at 65°C. A 10µl aliquot of each digest was analysed by electrophoresis on a 4 % agarose gel. If the product was cut to generate a distinct 104 bp and a faint 24 bp (often not visible) by *Sau* 3A and cut to yield a 122 bp by *Taq* 1, the sample was regarded as HTLV-I whereas if the product was not digested by *Sau* 3A but cut by *Taq* 1 to generate a 69 bp and a 53 bp, the sample was regarded as HTLV-II.

For each PCR, DNA extracted from C8166 cells (Hahn et al., 1983) and C1218M cell line (Clapham et al., 1984) were included as positive controls for HTLV-I and HLTV-II respectively.

Primer	*Sequence*	*expected size (bp)*
HIV-2 LTR outer		
Sense (U3 LTR)	5'-TAACCAAGGGAGGGCATGGG-3'	
Antisense (R LTR)	5'-TGGTGAGAGTCTAGCAGGG-3'	181
HIV-2 LTR inner		
Sense (U3 LTR)	5'-AGGAGCTGGTGGGGAACGCCCT-3'	
Antisense (R LTR)	5'-Biotin-AACACCCAGGCTCTACCTGCT-3'	141
HIV-1 LTR outer		
Sense (R LTR)	5'-ACCAGRTYTGAGCCTGGGAGCT-3'	
Antisense (U5/PBS)	5'-CCTGTTCGGGCGCCACTGCTAGAGATTTT-3'	177
HIV-1 LTR inner		
Sense (R LTR)	5'-TGAGCCTGGGAGCTCTCTGGCT-3'	
Antisense	5'-CTGAGGGATCTCTAGWYACCAGAGT-3'	120
HTLV-I *tax/rex* outer		
Sense	5'-CGGATACCCAGTCTACGTGT-3'	
Antisense	5'-GAGCCGATAACGCGTCCATC-3'	159
HTLV-I *tax/rex* inner		
Sense	5'-GTGTTTGGCGATTGTGTACA-3'	
Antisense	5'-CCATCGATGGGGTCCCA-3'	128

Table 2.1. Primer sequences for nested PCR for HIV-1, HIV-2 and HTLV provirus detection
Underlined bases represent overlapping sequences in nested reactions. Degeneracies incorporated at variable regions of the HIV-1 LTR were R=purine (A/G), Y=pyrimidine (C/T), and W=wobble (AGT)

2.c.4. Quantitative DNA PCR

i. Quantification of HIV-1 and HIV-2 proviral DNA

For quantification of HIV proviral DNA, primary PCR was conducted as described in section 2.c.2 except that 5μl (0.3μg) of DNA extract was used for quantification of HIV-1 proviral DNA. 1μl of primary PCR product was amplified by a modified secondary PCR. The secondary PCR was performed in a reaction volume of 50μl, containing 1 unit of recombinant *Pfu* DNA polymerase (Stratagene, Ltd., Cambridge, U.K.), *Pfu* DNA polymerase reaction buffer {20mM Tris-HCl [pH 8.8], 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 0.1mg/ml nuclease-free bovine serum albumin (BSA)}, 10pmol of inner primers of which 5' end of antisense primer was biotinylated, a 5μM of each of the three nucleotides (dGTP, dTTP, and dCTP) and 5μCi of ^{35}S-radiolabelled dATP (DuPont, Boston, MA). 30μl of the PCR product incorporated with radiolabelled ATP was diluted in a 30μl of PBS/0.05% Tween 20 and captured in streptavidin-coated microwells (Maxisorp U16, Nunc, Denmark) which were prepared by coating with streptavidin (5μl/ml) in coating buffer {10mM Tris-HCl, [pH 7.6]} over night, followed by blocking with coating buffer containing 0.1% BSA. After incubation at room temperature for 45 minutes, the plate was washed thoroughly 10 times with PBS / 0.05% Tween 20. Then 0.15M NaOH was added to strip the nonbiotinylated strand of secondary PCR product which was counted with the LKB Betaplate counter.

In order to estimate a number of copies of proviral DNA in a patient's sample, a series of external standards containing 3, 15, 80, 400, 2000 and 10,000 HIV-2 provirus copies per 10μl of DNA lysate was generated by serially diluting DNA extract from a C8166 cell line chronically infected with HIV-2_{CBL-22} (MRC AIDS Reagent Project, Potters Bar, U.K.) with DNA extract from HIV-uninfected C8166 cells for quantification of HIV-2 proviral DNA. For quantification of HIV-1 proviral DNA, a series of external standards containing

5, 36, 170, 2100, 10000 HIV-1 provirus copies per 5µl of DNA lysate was made by serially diluting DNA extracted from a 8E5 cell line (MRC AIDS Reagent Project, Potters Bar, U.K.) with DNA extract from HIV-uninfected C8166 cells. A series of external standards were included in each assay and a standard curve was drawn for each experiment (Figure 2.3). The number of copies in the standards was determined by limiting dilution analysis with nested PCR. Each samples was tested in duplicate, and if the variation in the duplicate was ≥ 10 fold, the sample was re-tested. On the assumption that the $CD4^+$ cell fraction harboured most viruses, the number of proviral copies per 10^5 $CD4^+$ cells was calculated by dividing proviral copies/10^5 PBMCs by $CD4^+$ cell percentage. If the sample was positive by qualitative PCR but the proviral copy number was <3 copies, the sample was assigned a value of 2.

Inter-assay variation was assessed by including 4 aliquots of the quality control in each experiment. Inter-assay variation throughout the experiments was less than two fold. In order to avoid the inter-assay variation in the evaluation of viral load change within a patient, all samples from one patient were tested in one experiment.

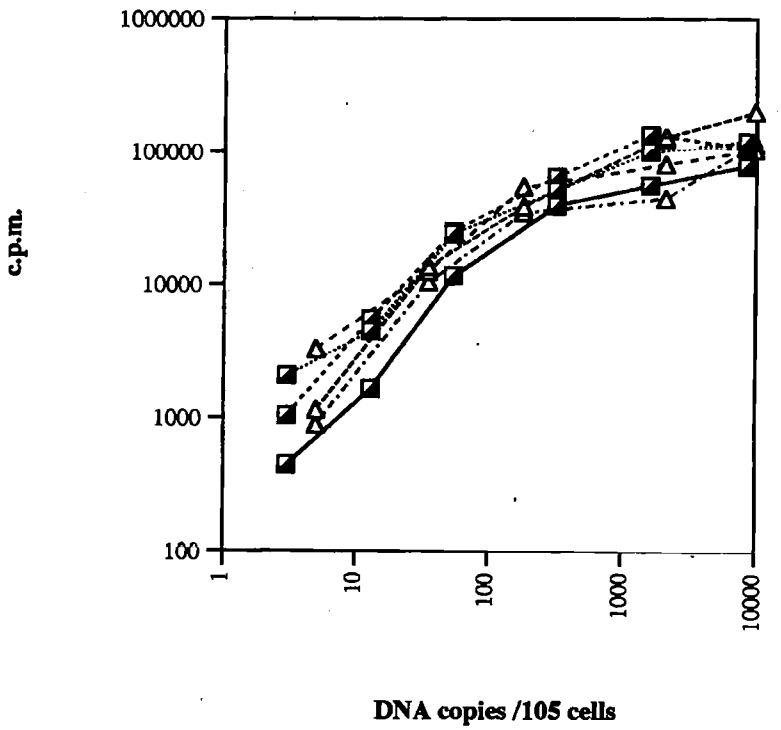

Figure 2.3. Standard curves for quantitative DNA PCR assays.
Three representative standards curves for HIV-1 quantitative PCR assay are shown in open triangles and for HIV-2 quantitative PCR assay shown in shaded squares; c.p.m., count per minute.

ii. Quantification of HTLV-I proviral DNA

In this study the levels of HTLV-I proviral DNA were analysed by limiting dilution analysis as follows: sample DNA was diluted in PCR-compatible DNA lysis buffer {50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.45 % NP40, 0.45 % Tween-20} in five fold steps using a Gilson positive displacement pipette. Each time the diluted DNA was transferred, a new piston and a capillary were used. At each dilution qualitative nested PCR was performed with *tax/rex* primers as described in section 2.c.3. To determine the end-point dilution, aliquots of diluted sample around the end-point were tested in quadruplicate. If two or more of 4 tests were positive, the dilution was regarded as positive and the reciprocal value of the end-point dilution was expressed as titre of HTLV-I provirus (copies / 1 μg DNA). In order to investigate the frequency of HTLV-I-infected cells in two subjects with an extremely high proviral load, 1.5×10^6 cells / ml of PBMCs suspension was serially diluted in five-fold steps by transferring 200 μl in Eppendorf tubes. The diluted cells were spun at 10,000 rpm for 3 minutes and after removing the supernatant completely, 150 μl of lysis buffer with proteinase K was added to extract DNA from the pellet at each dilution following the method described in section 2.c.1. The crude DNA lysate of each dilution was tested by nested PCR to determine the end-point as described above. To ensure the presence of cellular DNA at the end-point dilution, nested PCR for β-globin gene were applied.

2.c.5. Quantitative RNA PCR

i. Extraction and reverse transcription of HIV RNA

Viral RNA was extracted from heparinised plasma, which had been stored at -70°C or in liquid nitrogen, using a silica-based guanidinium isothiocyanate extraction method (Boom et al., 1990). A 200μl of plasma sample was mixed with 800μl of lysis buffer {100mM Tris-HCl [pH 6.4], 5M guanidinium thiocyanate, 20mM EDTA, Triton X-100} then 50μl of silica was

added. RNA bound silica was washed five times: (X2) in 5M guanidinium wash buffer {100mM Tris-HCl [pH 6.4], 5M guanidinium thiocyanate}, (X2) in 70% ethanol, and (X1) in acetone. The final silica pellet was dried on a hot-block at 56°C for 10 minutes before eluting RNA in 50µl of nuclease-free water. A 10µl of RNA extract was subject to *in vitro* reverse-transcription (RT) to synthesise first-strand cDNA in a 20µl of reaction volume, containing 50mM Tris-HCl [pH 8.3], 75mM KCl, 3mM MgCl2, 0.1M DTT, 10mM of each dNTP, 150-200ng per reaction of HIV-2 RT primer which target the primer binding site and its immediately upstream in conserved regions (Table 2.2) and 200 units of Superscript II reverse transcriptase (Gibco-BRL, Life Technologies). The RT reaction mix was incubated at 45°C for 50 minutes.

ii. Detection and quantification of HIV-2 cDNA

A 5µl of cDNA was amplified, in duplicate, in a reaction volume of 50µl containing 1 unit of *Pfu* DNA polymerase, *Pfu* DNA polymerase reaction buffer, 200µM of each dNTP, and 10pmol of outer primers for HIV-2 R/U5 LTR (Table 2.2) with 40 heat-cycle {94°C/2 minutes denaturation then 94°C/30 seconds denaturation, 50°C/30 seconds aneal, and 72°C/30 seconds extension (40 cycles) and 72°C/2 minutes further extension}. For the detection of cDNA, secondary PCR was conducted in a reaction volume of 25µl, which contains 1 unit of *Pfu* DNA polymerase, *Pfu* DNA polymerase reaction buffer, 200µM of each dNTP, and 5pmol of inner primers for HIV-2 LTR with 25 heat-cycle {94°C/30 seconds, 50°C/30 seconds, 72°C/30 seconds (25 cycles) and 72°C/2 minutes}. The product was run on a 1.5% agarose gel. If a single clear band was seen around 157 bp, it was regarded as positive.

The quantity of PCR product was determined using a chemiluminescence-based enzyme linked oligonucleotide assay (ELONA) (Whitby & Garson 1995). A 5µl of PCR product

was diluted with 95µl of sample diluent buffer {PBS, 5% casein, 0.5% Tween-20} then placed on a streptavidin-coated black plate (Life Science International, Hampshire) to capture the PCR product. After being incubated for 45-60 minutes at room temperature, the plate was washed five times with TTA buffer {10mM Tris-HCl [pH 7.5], 0.5% Tween-20, 0.01% sodium azide}. 100µl of 0.15M NaOH was added to denature the double strand PCR product then the unbiotinylated strand was washed away by five times wash with TTA. A single-stranded PCR product was then hybridised with 100µl of Alkaline phosphatase conjugated probe in reaction buffer {10X SSC, 5% casein, 0.5% Tween-20} at 45°C for 60 minutes. Excess probe was removed by washing five times wash with TTA before adding 100µl of substrate Lumi-Phos-530 (Lumigen). After incubation for 1 hour at room temperature in the dark, photon emissions were counted by a luminometer (Canberra-Packard, UK) or (Luminoskan, Labsystem, Finland).

To estimate the number of RNA copies per ml of sample plasma, external controls were included for each assay which were obtained by aliquoting a large stock of plasma samples from HIV-2-infected patients. The number of RNA copies / ml of external controls was estimated by determining the number of cDNA copies by limiting dilution analysis with a nested HIV-2 PCR and converting the number of cDNA copies / 5µl of cDNA to RNA copies / ml of plasma, using a conversion factor of 500 (Berry et al., manuscript submitted). This conversion factor was derived from repeated measurements of HIV-1 plasma RNA levels and is based upon the assumption of equal sensitivity for HIV-2 as the principle of the tests is the same. The number of cDNA copies / 5µl of cDNA derived from HIV-1 plasma samples was determined by limiting dilution analysis using a nested HIV-1 RNA PCR; the conversion factor was then calculated by dividing the number of HIV-1 RNA copies / ml determined by a commercial quantitative PCR assay for HIV-1 (Amplicor, Roche) by the result obtained from the limiting dilution method. Titrated

plasma controls of 200,000 and 80,000, 20,000 and 2000 RNA copies per ml or equivalents were used for each assay and a standard curve was drawn (Figure 2.4). The lower level of detectability was calculated to be 500 copies of plasma RNA / ml which corresponds to 1 copy of cDNA / 5µl cDNA. A working cut-off value of 500 copies per ml was used. If the sample was positive by nested PCR but less than 500 RNA copies per ml by quantitative PCR, the sample was assigned a value of 500. If the sample was negative by nested PCR, the sample was assigned a value of 250.

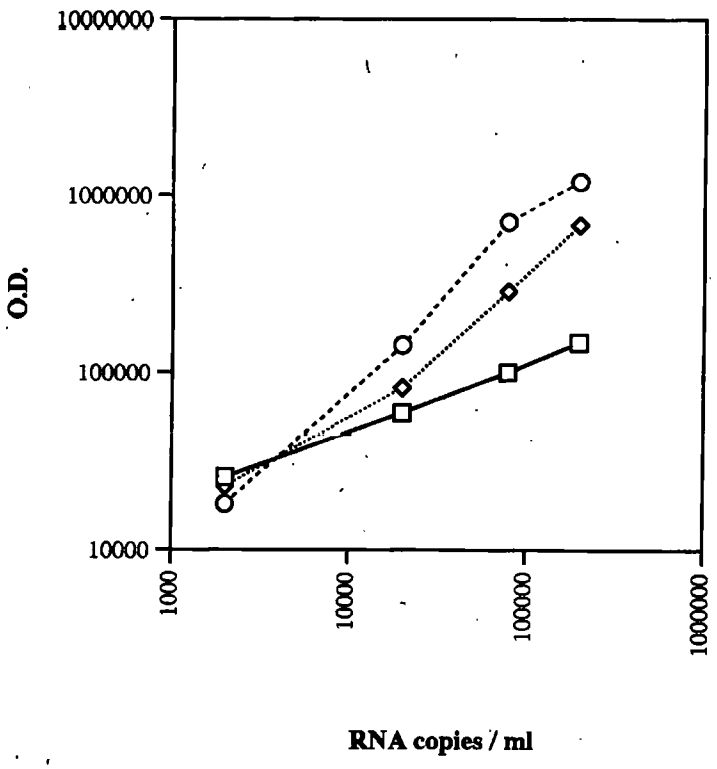

Figure 2.4. Standard curve for quantitative RNA PCR assay.

Primers	*Sequence*	*expected size (bp)*
HIV-2 R/U5 LTR outer		
Sense (R LTR)	5'-ATTGAGCCCTGGGAGGTTCTCTCCA-3'	
Antisense (RT primer) (U5/PBS)	5'-TTCGGGCGCCAACCTGCTAGGGATTTT-3'	
HIV-2 R/U5 LTR inner		
Sense (R LTR)	5'-AGCAGGTAGAGCCTGGGTGTT-3'	157
Antisense (U5 LTR)	5'-ACCAGGCGGCGACTAGGAGAGAT-3'	

Table 2.2. Primer sequences for RT reaction and nested PCR for HIV-2 viral RNA detection.

2.d. Cytotoxic T-lymphocyte assay.

2.d.1. Preparation of target cells.

An Epstein-Barr virus (EBV) transformed autologous B lymphoblastoid cell line (BCL) was established from each subject and cryopreserved before the use. When the second sample was taken for the bulk-culture, the BCL from the subject was thawed and cultured in RPMI 1640 plus 10% Foetal Calf Serum (FCS)(Life Technologies, U.K.) for at least 7 days. Target cells were infected with various recombinant vaccinias expressing the HIV-2 {gag(ROD), pol(ROD) or nef(BEN)} genes or VSC8 vaccinia expressing an irrelevant protein (β-galactosidase), at a multiplicity of infection of 3:1 for 1 hour and cultured in RPMI 1640 plus 20% FCS for 16 hours. The cells were labelled with ^{51}Chromium (100mCi $Na_2{}^{51}CrO_4$ Amersham U.K.) for one hour then washed three times.

2.d.2. Preparation of effector cells.

Fresh PBMCs were separated on Ficoll gradient from 20 ml of preservative free heparin treated blood within two hours after the venipuncture. One eighth of the PBMCs were stimulated for 24 hours with phytohaemagglutinin(PHA, Wellcome U.K.)(2μg/ml) and washed once before being added to the unstimulated PBMCs in order to boost HIV specific CTL. This "Bulk culture" was maintained in RPMI1640 plus 10% FCS in a 25cm^2 T-flask (Cell-cult, Sterilin, U.K.) in an up-right position for 7 days when 10% of Interleukin-2 (lymphocult T, Biotest, U.K) was added. CTL activities were tested at day-7 in 17 subjects and at day-14 in all subjects. As responses were highest and most frequent at day-14, these results were used in the analysis.

2.d.3. ^{51}Chromium release assay.

^{51}Chromium (^{51}Cr) labelled target cells were plated out at 5000 cells/well in 96 well U-bottomed plate (Sterilin, U.K.). In two HIV-2 infected subjects {F11, F19} with a low CD4%, 2000 target cells/well were used. The effector cells were added in duplicate at 60:1,

30:1 and 15:1, effector:target (E:T) ratio. Controls for background (target cells with media alone) and maximal (with 5% triton X 100) ^{51}Cr release were plated in quadruplicate. After incubating the plate for 5 hours, radioactivity in the supernatant was counted using the LKB Betaplate counter. Specific lysis(%) was calculated from the formula:

(experimental count - background) x 100 / (maximal count - background).

The specific lysis(%) to control vaccinia was subtracted from the specific lysis against HIV protein to describe HIV-specific lysis. HIV-specific lysis of 10% or more was regarded as positive. Background ^{51}Cr release was less than 30% of maximal count in all experiments.

2.e. Miscellaneous methods.

2.e.1. CD4/CD8 subset analysis.

Analysis of lymphocyte subsets has been done with fluorescence activated flowcytometry (FACScan, Becton-Dickinson, Belgium). For the community-based study in Guinea-Bissau, cells in EDTA-treated whole blood were stained with monoclonal antibodies (Becton-Dickinson, Belgium); red blood cells were lysed and white blood cells were stabilised and fixed using a commercially available reagents (ImmunoPrep, Coulter Electronics Ltd. England) and a machine at the field laboratory (Q-Prep®, Coulter). Samples were then transferred in a cool box to the main laboratory in The Gambia within a week, where FACScan analysis was done. For the hospital-based study in The Gambia, cells in EDTA blood were stained with the same antibodies but red blood cells were lysed with commercially available lysis reagents (FACS® brand lysing solution, Becton Dickinson, Belgium) and fixed with 0.5% formaldehyde.

Due to incomplete erythrocyte lysis and residue of cell debris, some samples were occasionally contaminated with a considerable amount of unlysed erythrocytes or debris which were counted as cells by FACScan and caused serious underestimate of CD4 and CD8 count. To minimise this problem, if sum of CD4% and CD8% was less than 45%, the data

was excluded from the analysis. This verification is particularly necessary when an accurate slope of CD4 decline is to be estimated.

Subjects were divided according to the new Center for Disease Control classification into 3 categories based on their % of CD4+ cells: low < 14%, medium 14 - 28%, high ≥ 29% (Center for Disease Control and Prevention 1994).

Total white blood cells per µl of blood and percentage of lymphocyte were manually determined.

2.e.2. Examination of blood film.

The presence of *Plasmodium falciparum* parasitaemia was determined by reading 100 high-power fields of Giemsa-stained thick blood film by one experienced laboratory technician who was masked from all the other laboratory results. If any number of asexual *P. falciparum* was found, the slide was regarded as positive for parasitaemia.

2.e.3. Other laboratory tests.

Plasma samples were tested for *Treponema pallidum* haemagglutining antibodies (TPHA) (Microsyph, Porton, Cambridge, Newmarket, U.K.) and for Rapid Plasma Reagin (RPR) (Becton Dickinson, Cockeysville). Using commercial tests, active syphilis infection was diagnosed if both TPHA and RPR tests were positive.

Chapter 3.

A community-based study of HIV-2 proviral load in a rural village in West Africa.

3.a. Introduction.

Previously Berry *et al.*, using quantitative polymerase chain reaction (PCR), reported that HIV-2 proviral load in symptomatic patients attending the MRC clinic in The Gambia was similar to that of HIV-1-infected symptomatic patients (Berry *et al.* 1994). Another study which used tissue-culture methods, has suggested that the level of infectious virus in plasma or cells in HIV-2-infected patients with a relatively high CD4 count was lower than that found in similar group of HIV-1-infected subjects (Simon et al. 1993). Therefore it was postulated that a low viral load, perhaps as a result of high levels of suppressive immunomodulation, may account for the better prognosis of HIV-2-infected individuals (De Cock et al. 1993, Marlink et al. 1994). However both previously published studies on HIV-2 viral load were hospital-based study and HIV-2 viral load in a community-based unbiased HIV-2-infected population has not been studied yet.

A community-based study in a rural village in Guinea-Bissau indicated that high prevalence of HIV-2 was seen throughout all age-groups including a substantial number of old people (≥ 60 years) and that the majority of infected individuals had a CD4% of ≥ 14% and no HIV-related symptoms (Wilkins et al. 1993, Ricard et al. 1994). Moreover two years of follow-up of these individuals showed that the mortality rate among HIV-2 infected individuals was only three-fold higher than that in age and sex matched HIV-uninfected individuals and that the mortality rate was not different between HIV-2-infected and HIV-uninfected individuals if they were 55 years or older (Ricard et al., 1994). This study provided an opportunity to investigate HIV-2 proviral load in these healthy HIV-2-infected individuals.

3.b. Results.

i. Study subjects.

Between January and May 1991, 132 HIV-2 seropositive individuals and 160 age and sex matched HIV seronegative individuals were recruited in a prospective cohort study in Guinea-Bissau (Chapter 2). Cryopreserved cells were available from 127 HIV-2 seropositive samples which were initially tested for the presence of HIV-2 provirus by qualitative PCR; 121 of these positive samples which contained more than 5 x 10^5 cells were considered suitable for a quantitative PCR assay. Sixty samples were chosen from the controls in order to test the specificity of PCR. The population in the study village has been followed with annual censuses, the last being conducted in March 1995.

ii. Detection and measurement of HIV-2 proviral load.

HIV-2 proviral DNA was detected in 125 (98.4%) of the 127 seropositive PBMC samples by qualitative PCR. Of the two samples which did not contain HIV-2 sequences, the samples were consistently negative in 20 experiments and the subjects have not been included in the analysis. None of the 60 samples from HIV seronegative controls were found to contain HIV-2 sequence by nested PCR. The geometric mean (95% C.I.) proviral load of the specimens from 121 HIV-2-infected subjects which contained at least 5 x 10^5 cells, was 35.5 (25.7, 49.0) proviral copies / 10^5 PBMC. The geometric mean (95% C.I.) proviral load for 104 subjects with valid CD4 data was 124.3 (86.0, 179.6) copies / 10^5 $CD4^+$ cells.

There was a considerable variation in proviral load, ranging from 2.4 to 16045 copies / 10^5 $CD4^+$ cells among the study population (Figure 3.1). An inverse relationship between proviral load expressed as copies / 10^5 PBMC or copies / 10^5 $CD4^+$ cells and

CD4% was found ($r = -0.22$, $p = 0.02$ or $r = -0.42$, $p < 0.001$ respectively). The geometric mean (95% C.I.) for subjects with high (≥ 29%), medium (14 - 28%) or low (< 14%) CD4% was 85.2 (51.9, 140.1) (n = 55), 166.6 (94.9, 292.3) (n = 46) and 1385.7 (86.1, 22,311.1) (n = 3) proviral copies / 10^5 $CD4^+$ cells respectively.

iii. Proviral load in relation to age

No significant trend was noticed between age and the number of proviral copies / 10^5 $CD4^+$ cells ($r = -0.06$, $p = 0.5$). Proviral load was stratified into three age-groups. The geometric mean (95% C.I.) proviral load of those less than 40 years, those 40 - 59 years and those ≥ 60 years was 151.8 (66.2, 348.3) (n = 26), 113.8 (71.1, 181.9) (n = 48) and 120.3 (52.0, 278.4) (n = 30) proviral copies / 10^5 CD4+ cells respectively. Considerable variation was observed in all age-groups and the mean viral load was not significantly different in three age groups (Figure 3.2).

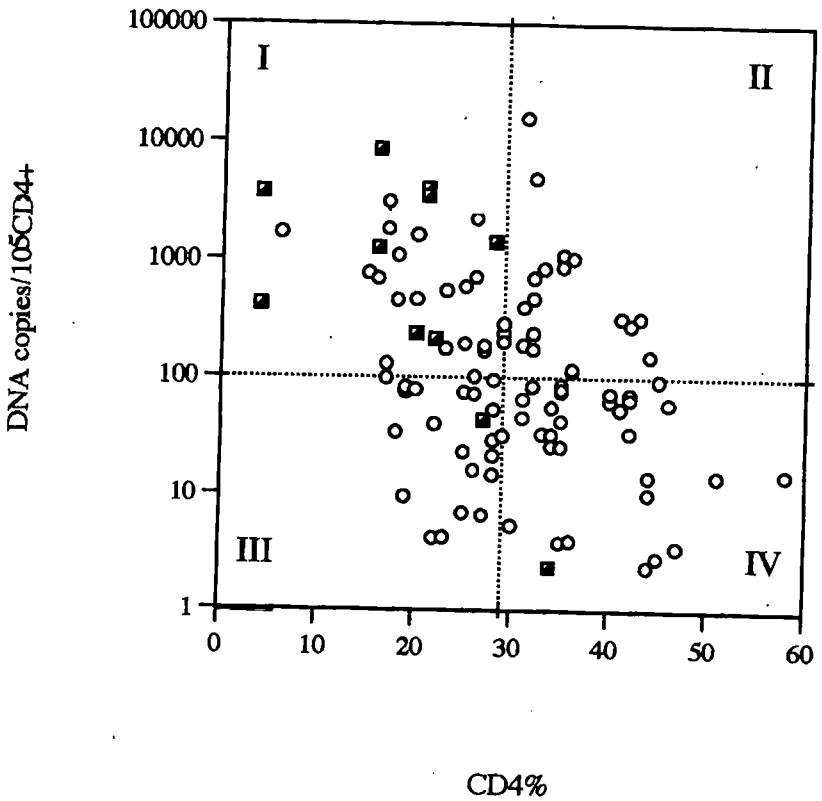

Figure 3.1. HIV-2 Proviral load and CD4% in 104 villagers.
The eleven subjects who died are indicated by the shaded square dots; the subjects have been divided into 4 categories (I-IV) by the dotted lines which represent a CD4% of 29% and a provial load of 100 copies / 10^5 CD4+ cells.

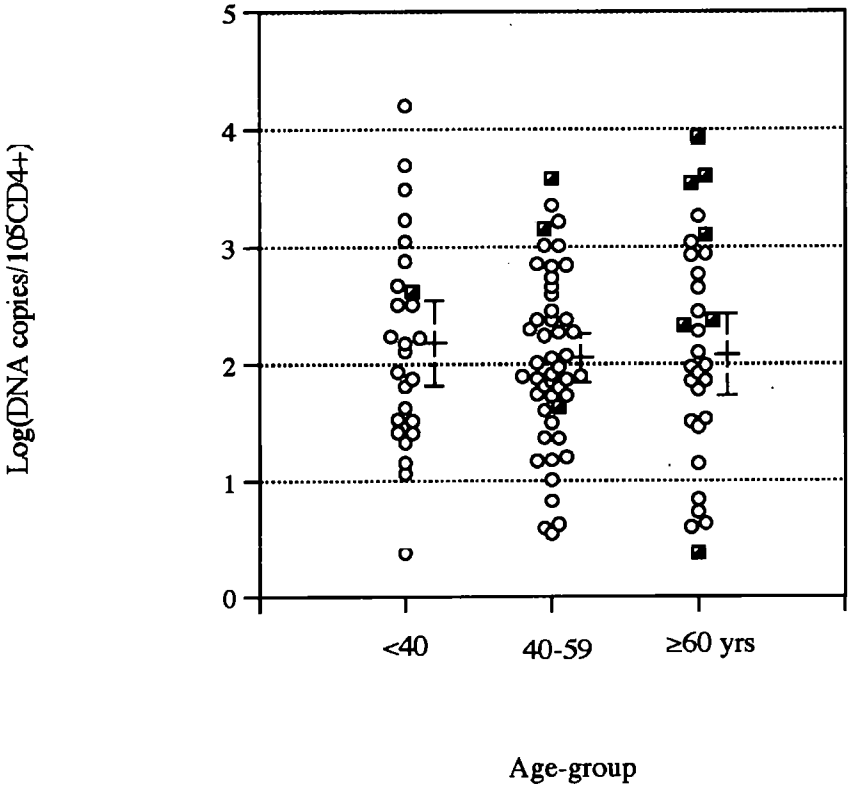

Figure 3.2. HIV-2 proviral load in three age groups.
Geometric means and 95% confidence intervals are indicated by horizontal bars; the eleven subjects who died are indicated by the shaded square dots.

iv. Proviral load in relation to co-infections

Many subjects were also infected with active syphilis, malaria or HTLV-I. Active syphilis was diagnosed by TPHA and RPR serology, malaria infection by Giemsa-stained thick blood film, and HTLV-I infection by a nested PCR with restriction enzyme analysis. Typing of restriction digests of PCR products showed that all HTLV PCR positive samples were HTLV-I (Figure 3.3). The subjects were stratified according to the presence or absence of co-infection and the mean proviral load in each group is shown in Table 3.1. Proviral load did not differ significantly according to co-infection.

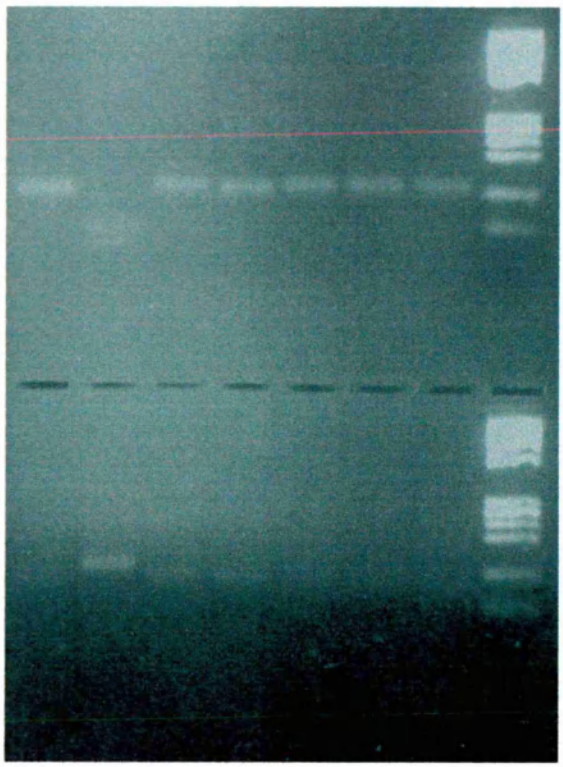

Figure 3.3. Restriction enzyme analysis of HTLV-I/II tax/rex PCR products.

Agarose gel (4%) electrophoresis of *Taq* 1 and *Sau*3a digests of secondary PCR products stained with ethidium bromide is shown. The upper half of the gel was loaded with *Taq*1 digests, the lower half with *Sau*3a digests. Reading from left to right, lane 1 contains digests of products derived from HTLV-I positive control (C8166), lane 2 contains digests of products derived from HTLV-II positive control (C1218M), lanes 3 to 7 contain digests of products from samples c32, c33, c67, c68, and c86. The extreme right lane carries the molecular-weight marker, phiX174/*Hae*III digest (the lowest two bands of the molecular weight ladder are 118bp and 72bp). The rest of samples showed the same pattern.

	Presence of infection	Absence of infection
Syphilis	142.2 (67.6, 299.2)	120.2 (78.0, 185.4)
(n)	(20)	(84)
Malaria	102.8 (36.8, 287.1)	145.5 (96.4, 219.8)
(n)	(18)	(80)
HTLV-I	131.8 (75.0, 231.7)	122.2 (77.4, 192.8)
(n)	(23)	(81)

Table 3.1.

Effects of co-infection with active syphilis, malaria and HTLV-I on HIV-2 proviral load.

Geometric means (95% confidence interval) copies / 10^5 $CD4^+$ cells are shown.

v. Proviral load in relation to mortality

Since the start of the cohort study until March 1995, 6 out of the 104 subjects with valid CD4 data had moved from the village, 87 subjects still lived in the village and 11 have died. The six subjects who moved have been excluded from the mortality analysis. Mean (range) duration of follow-up among subjects who died was 580 (29 - 1328) days. The age adjusted geometric mean (95%C.I.) proviral load of 11 subjects who died was 654.2 (181.9, 2352.2) copies / 10^5 CD4+ cells which was significantly higher than that of 87 subjects who survived which was 104.1 (70.4, 153.9) copies / 10^5 CD4+ cells ($p = 0.003$). The age adjusted mean (95% C.I.) CD4% of those who died was 19.5 (13.2, 25.9)% which was significantly lower than that of the survivors which was 30.3 (28.3, 32.3)% ($p < .001$). The subjects were stratified into four categories according to their proviral load and CD4% (Figure 3.1). Nine out of 28 subjects in category-I ($\geq$ 100 copies / 10^5 CD4+ cells and CD4% < 29%) died compared to 0/20 in category-II ($\geq$ 100 copies / 10^5 CD4+ cells and CD4% $\geq$ 29%), 1/20 in category-III (< 100 copies / 10^5 CD4+ cells and CD4% < 29%), 1/30 in category-IV (<100 copies / 10^5 CD4+ cells and CD4% $\geq$ 29%). Age and sex adjusted odds ratio (95% C.I.) between the mortality in category-I and the other categories was 32.5 (4.0, 263.8) ($p <0.001$). Thus the mortality rate was significantly higher in category-I than that in the other categories. The exact cause of death could not be ascertained in any of the subjects who died.

3.c. Discussion

No other study has related viral load to mortality in HIV-2 infection. This study first showed that a high proviral load and a relatively low CD4%, but not a relatively low CD4% or a high proviral load alone, was related to mortality. Most studies of viral load have been based on patients attending hospital thus the real picture of viral load in a community setting is lacking. In this village, apart from an increased presence of

lymphoadenopathy in cases, there were no clinical differences between the HIV-2 infected cases and uninfected controls (Ricard et al. 1994). Thus the study population were generally asymptomatic. However the level of HIV-2 proviral load varied considerably and the mean was found to be high, even in the subjects with a high CD4% of ≥ 29%. Previously published studies using quantitative PCR have shown that among asymptomatic HIV-1-infected individuals the mean level of HIV-1 proviral load was around 50 copies / 10^5 PBMC or 100 copies / 10^5 CD4+ cells (Ferre et al. 1992, Bieniasz et al. 1993, Chervert et al. 1994). Hence the level observed in healthy HIV-2-infected villagers appears to be similar to that found in HIV-1-infected asymptomatic individuals, although there is no comparable community-based viral load study of HIV-1 from Africa.

These findings of HIV-2 proviral load do not support the previous hypothesis that asymptomatic individuals infected with HIV-2 live longer because they have a lower viral load than HIV-1 infected individuals (Simon et al. 1993). Hence the level of proviral load *per se* may not be the only factor accounting for the better prognosis of HIV-2 infection. SIVagm can be consistently isolated from its natural host, the African Green Monkey, which does not suffer from immune deficiency even though it has a viral load of 5 - 50 copies / 10^5 PBMC (Hartung *et al.* 1992). Likewise HIV-2 may develop a similar relationship to its host, so that the provirus can be maintained at a substantial level without destroying the immune system and killing the host as fast as that observed in HIV-1 infection. As proviral load measures both productive and latent infection, one explanation may be that in HIV-2 infection more $CD4^+$ cells are latently infected and fewer are actively producing viruses than in HIV-1 infection. This may result in a slower rate of destruction of $CD4^+$ cells for recent studies have shown that the virus-producing cells are killed within a much shorter period than latently-infected cells (Wei et al., 1995; Ho et al., 1995). Alternatively in HIV-2

infection a larger proportion of proviral genomes may be defective than in HIV-1 infection. It has been planned to measure the level of HIV-2 RNA in plasma, which is a better index of productive infection than proviral load, and may be important in understanding the differences in the pathogenesis of the two infections. The level of HIV-2 RNA in plasma and its relation to the disease progression are studied in Chapter 4.

Other infectious diseases which are common in the tropics were investigated to test whether they influenced proviral load. The data did not show any significant effect of malaria, active syphilis or HTLV-I co-infection on HIV-2 proviral load. However since malaria parasitaemia is thought to be transient and because most villagers have intermittent parasitaemia, it would be difficult to demonstrate the effect of malaria on proviral load. The effect of HLTV-I and malaria co-infection is discussed again in Chapter 6.

The time course of the HIV-2 epidemic in this community and duration of HIV-2 infection in an individual subject are matters of speculation. However it is reasonable to assume that on average the duration of infection in the old subjects is longer than that in the young subjects. In this study we noted that the mean proviral load in the older age group ($\geq$ 60 years) was not significantly higher than that of the younger age group (under 40 years). Presuming that the virus in the older age group is as equally replication-competent as that in the younger age group, this suggests that in this population the proviral load of HIV-2 does not increase significantly with time. This may be a reflection of the better prognosis of HIV-2 infection.

3.d. Discussion of the 1996 follow-up survey

According to the most current census survey conducted in 1996, 6 more HIV-2-infected subjects died during 1995. Of those, HIV-2 proviral load data in 1991 was available in 4 subjects. On the contrary to the initial expectation, there was only one more death in the category of a high proviral load and a low CD4 count (Category-I); the rest of 18 subjects in this category have survived during the five years of follow-up. One subject who died, had a high proviral load and a high CD4 count (Category III) and two other subjects who died, had a low proviral load and a high CD4 count (Category IV) in 1991. These subjects may have had an increase in proviral load which caused the deaths. However since 7 deaths were notified among HIV-uninfected controls during 1995, which is more than the number of deaths among HIV-2-infected cases during the same period and also a similar cohort in Bissau showed that the mortality rate ratio decreased over-time (Poulsen et al., 1997), deaths observed later in the cohort may not be directly related to the HIV-infection.

Chapter 4.

Plasma RNA viral load predicts the rate of CD4 decline and death in HIV-2 infected patients in West Africa

4.a. Introduction.

Dichotomy in the rate of HIV-2-disease progression exists and it is important to investigate what factors determine the difference in the rate of disease progression (Chapter 1). Chapter 3 demonstrated that subjects with a high HIV-2 DNA load with a low CD4 count were more likely to die. However the 1996 follow-up data showed that several HIV-2-infected individuals with a high level of provirus, survived for long. As was discussed in general introduction, the level of provirus in peripheral blood lymphocytes does not necessarily reflect the rate of viral replication which is more directly reflected by the level of viral RNA in plasma. Later a number of papers have demonstrated that RNA viral load is a strong prognostic marker of HIV-1 disease progression (Mellors et al., 1995, 1996; Henrard et al., 1995; Phillips et al., 1996; Ruiz et al., 1996; de Wolf et al., 1997). Thus the measurement of plasma HIV-2 RNA viral load is essential to understand the pathogenecity of HIV-2. Recently Berry *et al.*, have developed an assay to measure HIV-2 RNA viral load in plasma and found in a cross-sectional observation that HIV-2 RNA load inversely correlated with $CD4^+$ count (Berry *et al.*, manuscript submitted). However the study did not relate HIV-2 RNA viral load with the disease outcome. Therefore this chapter examines whether HIV-2 RNA viral load is a better predictor for the rate of disease progression than HIV-2 DNA load. Since no longitudinal observations of HIV-2 RNA and DNA viral load over time have been reported, DNA and RNA viral load were longitudinally determined and compared between HIV-2-infected long-term slow/non-progressors and fast progressors.

4.b. Results.

i. Study subjects.

All study subjects were recruited from the clinical cohort at the MRC hospital, Fajara which is described in Chapter 2. From January 1991 to December 1992, 184 HIV-2 seropositive subjects who attended at the MRC hospital, were tested for $CD4^+$ and $CD8^+$ counts; 133 subjects had a CD4 ≥ 14% at the base line. The first sample collected during the first two years was used to measure base-line viral load. Since the aim of the first part of this chapter is to correlate HIV-2 viral load in earlier stages of infection with CD4 decline and the disease outcome, those patients with a base-line CD4%<14% or had less than three validated CD4 data or less than one year follow-up were excluded from the study. In June 1995 when the study to compare base-line HIV-2 proviral load with CD4 decline was closed, 38 subjects were selected as they had met the criteria for inclusion to this study: a base-line CD4% ≥14%, at least three validated CD4 data, and more than one year follow-up. In 1997 when an HIV-2 quantitative RNA PCR assay became available, the base line plasma samples of these 38 subjects were tested for RNA viral load. The mean slope of CD4% for each patient was determined by applying a linear regression line, assuming that CD4% decline linearly during the study period (Jaffar et al., 1997). In order to extend the period of follow-up, all available CD4% data and survival data up to March 1997 were used for this analysis. In order to observe HIV-2 RNA and DNA viral load longitudinally, 4 long-term slow/non-progressors were selected based upon the following criteria: those with CD4% remaining normal (>28%) and without HIV-related symptom for over 2500 days since HIV-infection was first diagnosed. Five fast-progressors were selected based upon criteria: those with CD4% decline faster than - 4% per year. Two of the long-term slow/non-progressors and two of the fast progressors who were recruited for this longitudinal observation after June 1995 have not been included in the over all analysis.

ii. Characteristics of Subjects.

The characteristics of the 38 subjects are summarised in Table 4.1. The subjects have been grouped according to their base-line DNA or RNA load in order to facilitate Kaplan-Meier survival analysis. The characteristics of selected 4 long-term slow/non-progressors and 5 fast-progressors are shown in Table 4.2.

iii. Base-line viral load in relation to CD4% slope

First, the relation between a base line HIV-2 viral load and a subsequent CD4 decline was analysed. Figure 4.1.a. shows that there was a significant inverse correlation between the base line RNA viral load and the subsequent CD4 decline ($r = -0.36$, $p=0.027$). The rate of CD4 decline per year increases by - 1.472 CD4% for each $\log_{10}$ increase in RNA viral load. However there was no significant relation between the base line DNA viral load and the subsequent CD4 decline (Figure 4.1.b) ($r = -0.19$, $p=0.26$).

Patient group	No. of subjects (death)	mean (range) age (years)	Proportion of asymptomatic patients	Median CD4% (range) at base line	follow-up time (PYO)	Mean (range) no. of observation
RNA viral load (copies / ml)						
< 500	19 (5)	38.5 (24, 67)	0.53	33 (23, 54)	81.6	6.5 (3, 11)
500- 50,000	13 (3)	31.8 (19, 59)	0.69	24 (14, 42)	59.7	6.0 (3, 9)
≥50,000	6 (5)	40.0 (17, 57)	0.20	20 (15, 30)	15.3	4.7 (3, 7)
DNA viral load (copies/10^5 PBMCs)						
< 50	23 (7)	37.8 (24, 67)	0.57	33.0 (17, 54)	100.9	6.4 (3, 13)
50 – 500	10 (3)	31.8 (17, 52)	0.40	25.5 (15, 42)	40.5	6.0 (3, 10)
≥500	5 (3)	37.3 (27, 57)	0.60	20.0 (14, 27)	19.5	4.7 (4, 9)

Table 4.1.
Characteristics of study patients when grouped by RNA or DNA viral load.
PYO, person-years of observation

Patients	Sex	Age at entry (yrs)	Duration of follow-up (days)	mean RNA viral load (copies/ml)	mean DNA viral load (copies/ 10^5CD4^+ cells)	Base line CD4%	CD4% decline (% per year)	last clinical status
Fast-progressors								
FNH	F	26	1061	86,080	21,655	20%	- 7.37%	Died
FFJ	F	35	559	89,025	4,754	30%	- 15.85%	Died
FFS	F	45	616	110,950	11,882	20%	- 5.95%	Died
FSJ	F	20	1688	383,200	1,930	21%	- 6.97%	Sympt
FNB	F	45	1448	111,350	5,465	23%	- 4.49%	Died
Long-term slow/non-progressors								
LLJ	M	50	3059	424	14	34%	- 1.41%	Asympt
LFS	F	35	2458	250	5	47%	- 0.06%	Asympt
LNF	F	19	2619	600	38	31%	1.6%	Asympt
IMM	F	30	2451	304	12	32%	- 0.06%	Asympt

Table 4.2.
Characteristics of 4 long-term slow/non-progressors and 5 fast-progressors

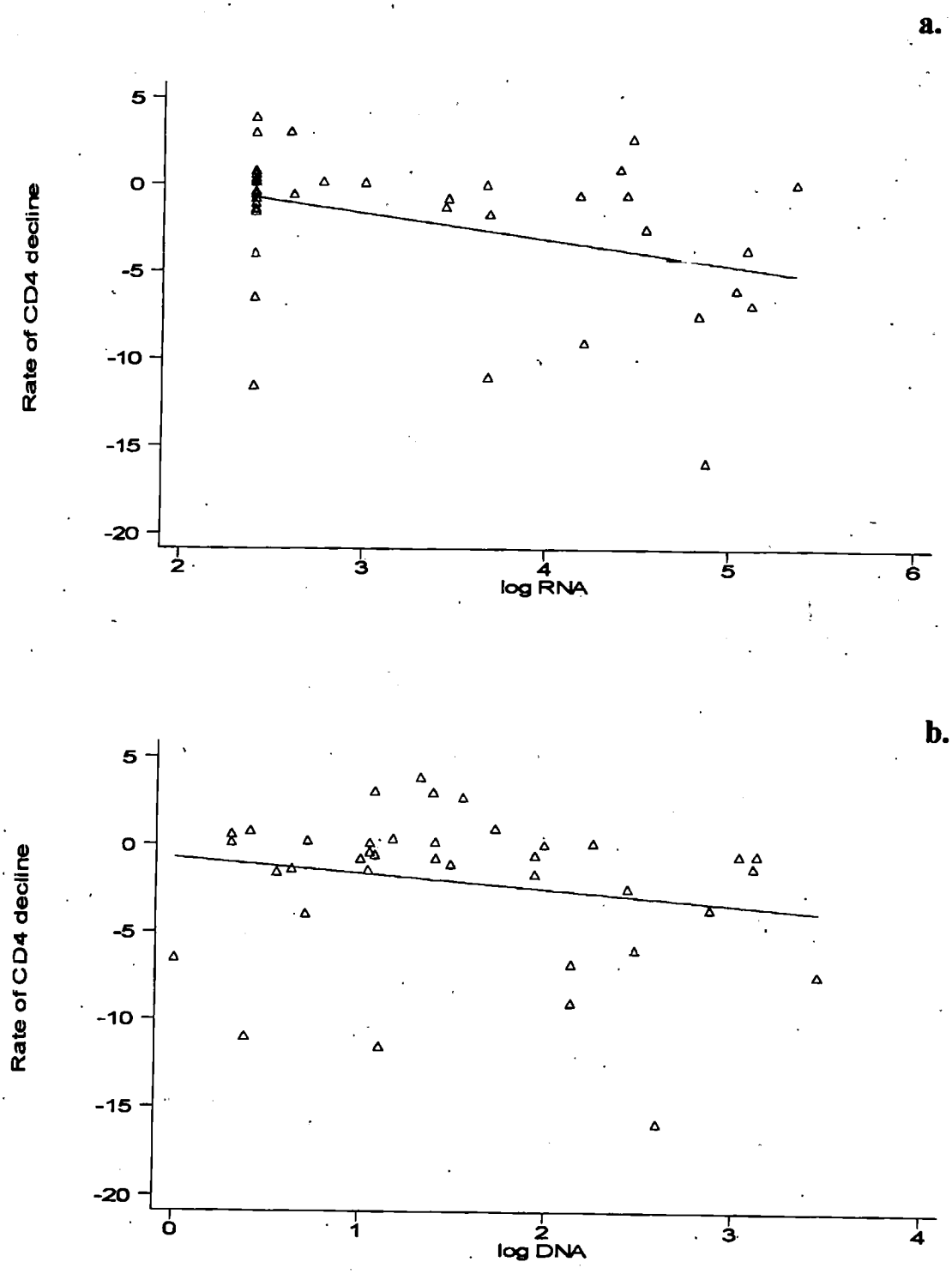

Figure 4.1. Comparison between HIV-2 viral load and $CD4^+$ cell decline.
The rates of CD4 decline (% per year) in 38 HIV-2-infected subjects are plotted in relation to their base line RNA (Figure 4.1.a) or DNA viral load (Figure 4.1.b).

iv. Survival analysis

The relation between survival and base line viral load when considered as a continuous variable was analysed using the Weibull regression model which assumes that the chance of death increases smoothly with time. After adjusting for CD4% and age, a strong correlation between survival and the base line RNA viral load was found; the Hazard ratio significantly increases at the rate of 3.01 (95% IC 1.5, 6.1) for each $\log_{10}$ increase in RNA viral load (p=0.002). The relation between survival and the base line DNA viral load was also significant but less strong; the Hazard ratio increase at the rate of 3.64 (95% CI 1.2, 10.8) for each $\log_{10}$ increase in DNA (p=0.02).

Kaplan-Meier survival estimates for each band of RNA and DNA viral load were also plotted in Figure 4.2.a. and 4.2.b. A clear difference in survival rate was noticed between the group with a low RNA viral load (<500 copies / ml) and the group with a high RNA viral load (≥50,000 copies / ml): the hazard ratio between these groups after adjusting for base-line CD4% and age was 52.3 (95% CI, 5.6, 483.3; p<0.0001). However the hazard ratio between the groups with a low and a medium RNA viral load (500 - 50,000 copies / ml) was not significant, being 3.1 (95% CI 0.53, 18.6) (p–0.21). When analysing the DNA viral load data, survival rates were not significantly different between the groups with a low DNA viral load (<50 copies / 10^5 PBMC) and the group with a medium (50-500 copies / 10^5 PBMC) or a high DNA viral load (≥500 copies / 10^5 PBMC). After adjusting for CD4% and for age, the hazard ratios between the groups with a low DNA viral load and with a high DNA viral load was 5.0 (95% CI 0.60, 41.8; p=0.14).

At least two deaths in the group with a low RNA viral load and one death in medium RNA viral load group were caused by HIV-unrelated disease: one was due to congestive heart failure and two due to hepatoma. Of those with a high HIV-2 RNA viral load, five patients have died within four years. All five appeared to have died with HIV-related diseases: three with recurrent chest-problems, one with septicemia and one AIDS which was diagnosed two years prior to her death.

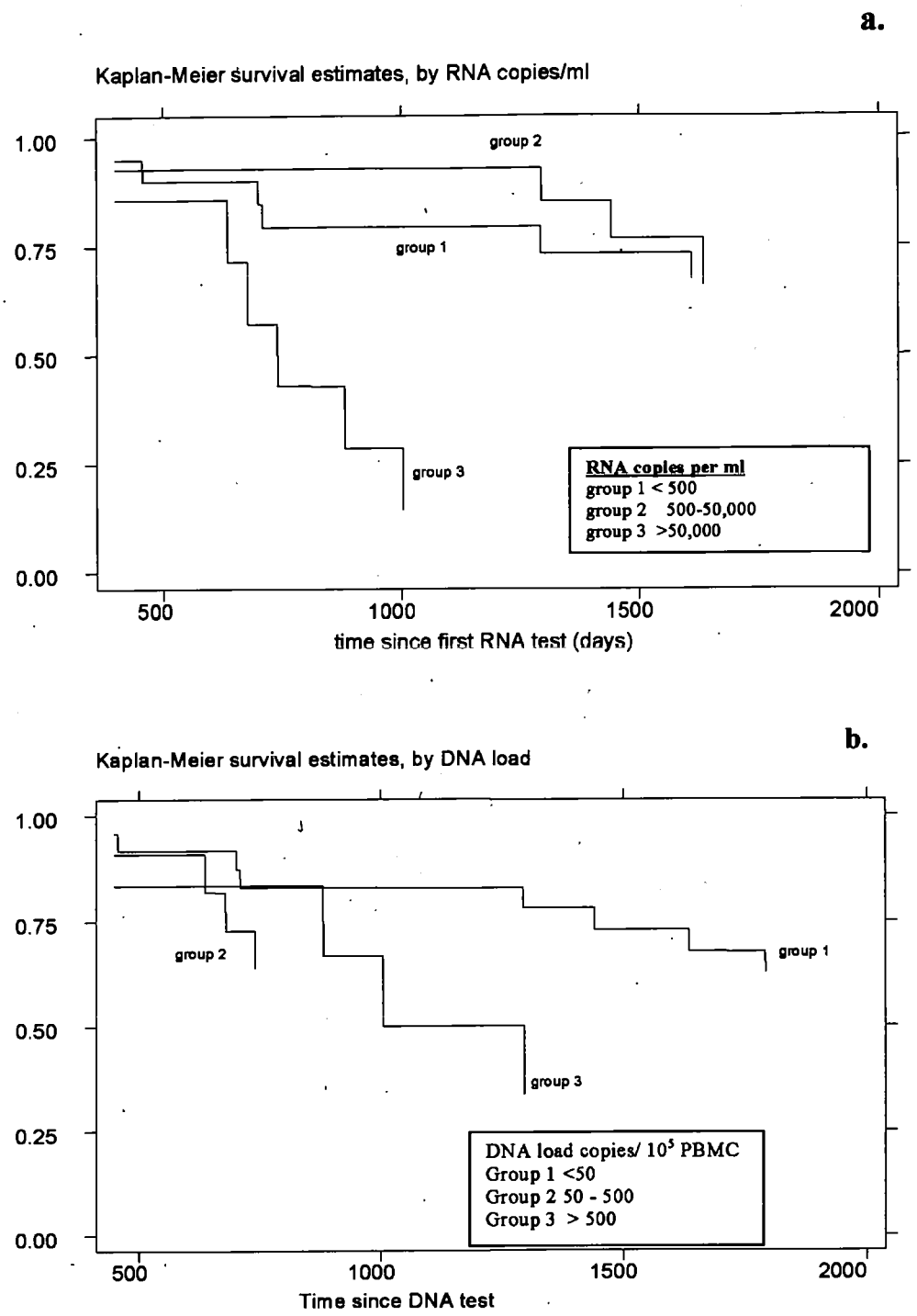

Figure 4.2. Kaplan-Meier survival analysis of 38 HIV-2-infected subjects according to RNA and DNA viral load.

v. Longitudinal observation of RNA and DNA viral load.

Profiles of CD4% and RNA and DNA viral load of 5 fast-progressors and 4 long-term slow/non-progressors are shown in Figure 4.3 and 4.4. Both RNA and DNA viral load are consistently high in the 5 fast-progressors and consistently low in the 4 long-term slow/non-progressors. The rate of CD4% decline and the mean RNA and DNA viral load in each fast-progressors and long-term slow/non-progessors are summarised in Table 4.2. The means of mean RNA and DNA viral load among 5 progressors were 156,121 copies/ml and 9,137 copies/10^5 $CD4^+$ cells respectively. These were more than two log higher than the means of mean RNA and DNA viral load among long-term slow/non-progressors which were 395 copies/ml and 17 copies /10^5 $CD4^+$ cells respectively.

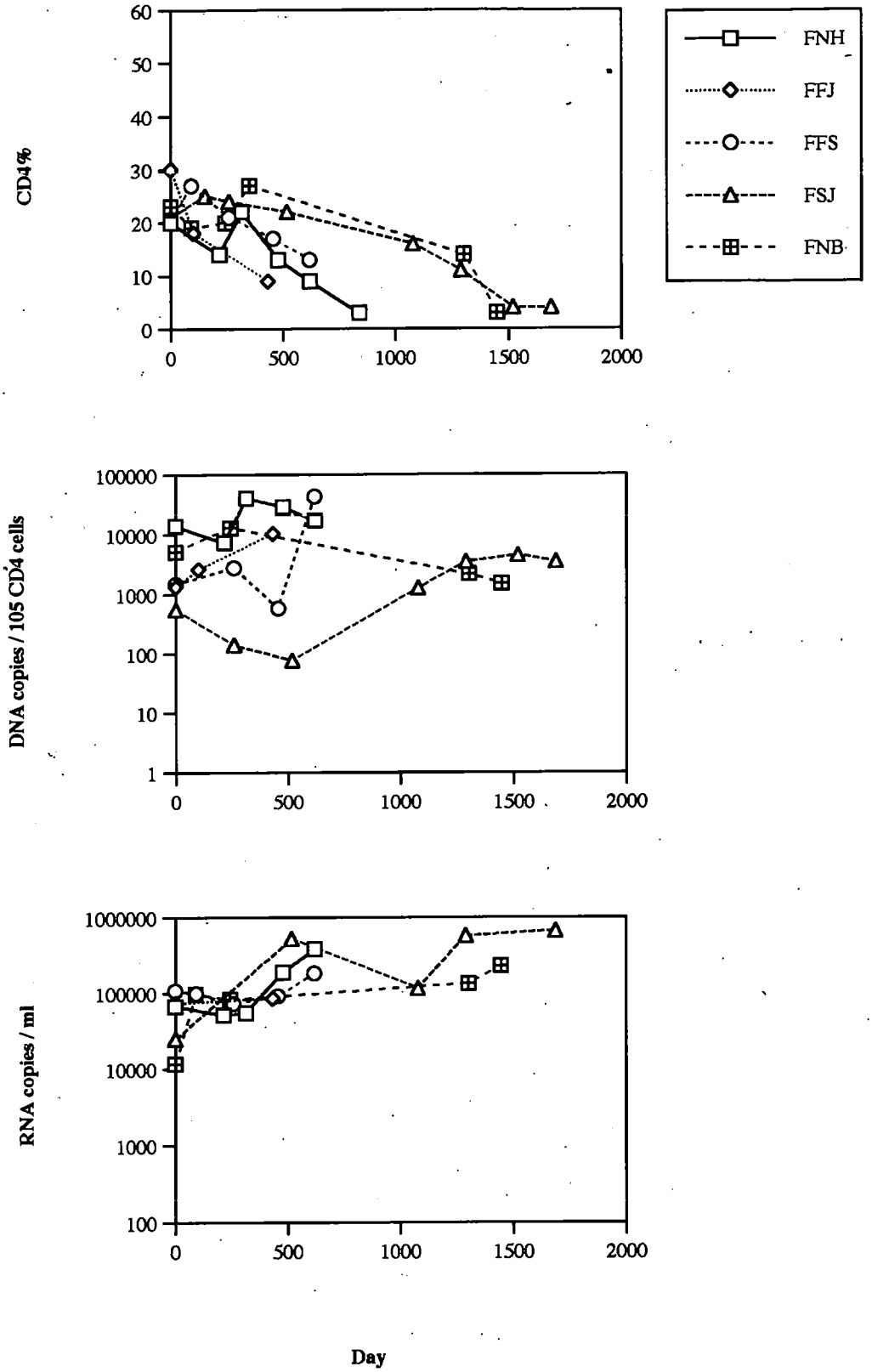

Figure 4.3. Profiles of CD4%, RNA, and DNA viral load of 5 fast-progressors

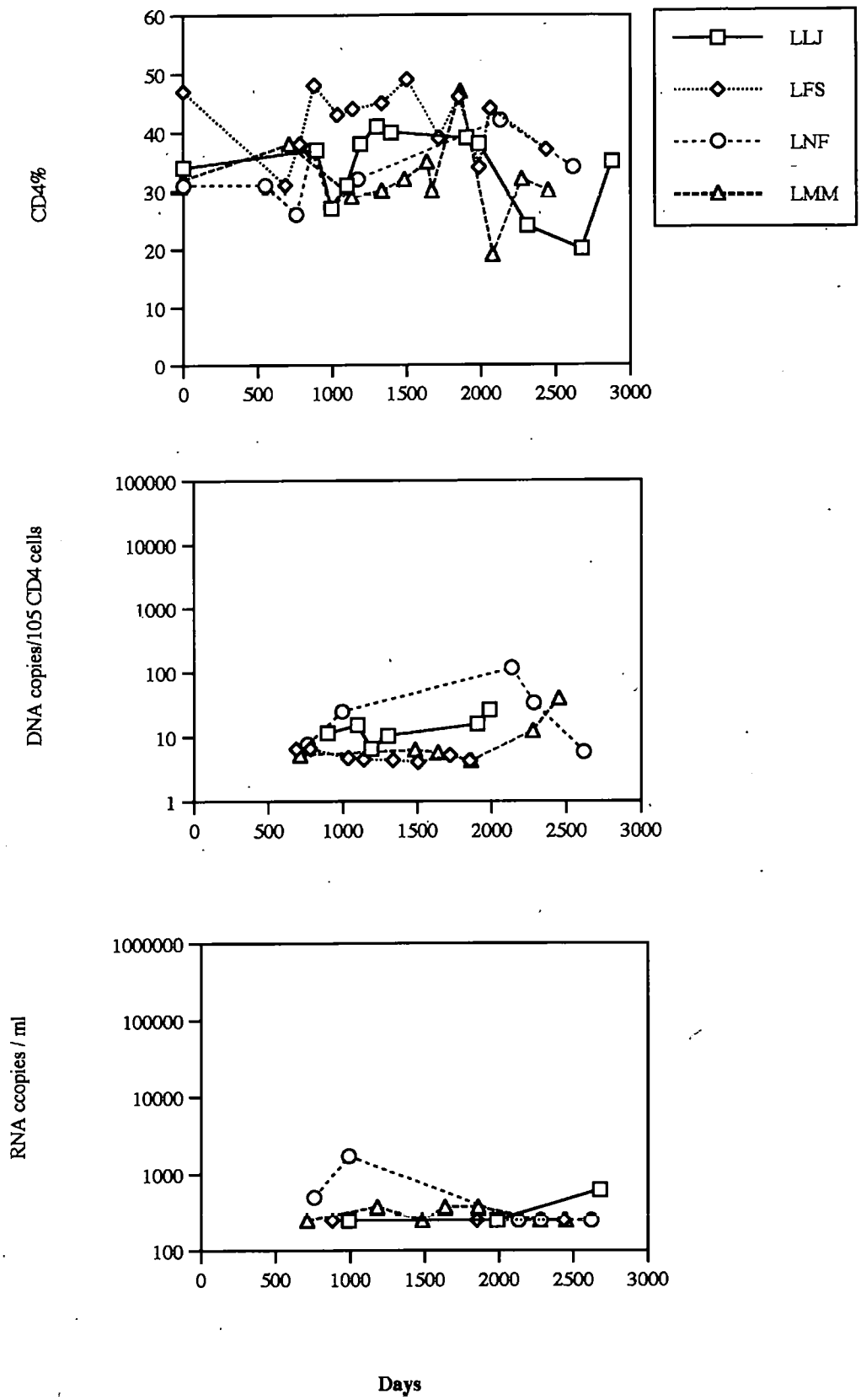

Figure 4.4. Profiles of CD4%, RNA, and DNA viral load of 4 long-term slow/non-progressors.

4.c. Discussion

The data demonstrates that a base-line HIV-2 RNA load significantly predicts the rate of disease progression as determined by $CD4^+$ decline or death but that base-line HIV-2 DNA load is a less sensitive predictor. This observation is compatible with conclusions drawn after a number of studies on HIV-1 viral load (Mellors et al., 1995, 1996; Henrard et al., 1995; Phillips et al., 1996; Ruiz et al., 1996; de Wolf et al., 1997) and implies that the mechanism of HIV-2 pathogenesis is similar to that of HIV-1. Therefore like HIV-1, a rapid decline of $CD4^+$ cell count is likely to be associated with a high rate of HIV-2 replication and a rapid turn-over of HIV-2 productively infected cells. The association between HIV-2 DNA load and the rate of disease progression was less obvious as some subjects had had relatively high levels of HIV-2 provirus for the low level of HIV-2 RNA in plasma. Perhaps in these individuals the majority of HIV-2 provirus was in a unintegrated form which is replication incompetent as has been demonstrated in HIV-1 (Chun et al., 1997).

There is a general view that HIV-2 infected individual progresses slower than HIV-1 infected individuals (Poulsen et al., 1989, 1997; Ridard et al., 1994; Whittle et al., 1994; Marlinks et al., 1994). However five HIV-2 progressors are described in this chapter who had had a high RNA viral load throughout the observation and a very rapid CD4% decline. As seroconversion time of the 5 HIV-2 fast-progressors was not known, the true length of HIV-2 infection is only speculative. However the mean age among the group with a very high RNA load ($\geq$ 50,000 copies /ml) was not significantly older than the other groups (Table 4.1) and at least three fast-progressors who are described in detail in Table 4.2 and Figure 4.3, were relatively young: 20, 26 and 35 years old. Therefore some of HIV-2 progressors are truly fast-progressing HIV-2 patients rather than those who started to progress as a consequence of longer incubation period of HIV-2 infection.

Four long-term slow/non-progressors with little evidence of CD4% decline were also identified and their RNA viral load were followed longitudinally. During the observation period of over 2500 days, RNA viral load was found to be consistently low as has been observed in HIV-1 long-term non-progressors (Chapter 1). This indicates that HIV-2 infected slow/non-progressors have established a steady relationship with the virus at a low rate of HIV-2 replication and this steady state relationship is one of the mechanisms which dictate a better clinical outcome in HIV-2 infection.

The reason for this dichotomy in the rate of HIV-2 replication and the rate of disease progression remains largely unknown. Various virological factors have been postulated including:. biological properties of HIV-2 such as *in vitro* virus replication and syncytia forming properties (Albert et al., 1990a; Schulz et al., 1990), chemokine receptor usage pattern may also be important as HIV-2 primary isolates from one of a fast-progressor were found to use broad chemokine receptors such as CCR1, CCR2b, CCR3, CCR4, CCR5 and CXCR4 (McKnight et al., manuscript submitted), HIV-2 viral genotype may be linked to mortality in a community-based study in a rural village (Grassly et al., manuscript submitted). Various host factors have also been postulated: HIV-2 specific cytotoxic T-lymphocyte (CTL) activities (Gotch et al., 1993; Chapter 5), neutralising antibodies (NA) against autologous viruses (Bjorling et al., 1993). Perhaps the host-virus inter-relationship must be important for determining the rate of disease progression. Therefore if certain individuals are infected with a certain viral genotype, it causes disease but the same strain does not necessarily cause disease at the same rate in different individuals.

Chapter 5.

HIV-2-Specific Cytotoxic-T-Cell activity is inversely related to proviral load

5.a. Introduction.

Cytotoxic T-cells (CTL) are thought to play an important role in controlling various viral infections (reviewed by McMichael & Walker, 1994). In chapter 1, it is discussed that in most HIV-1-infected asymptomatic individuals CTL can be demonstrated against the structural proteins Env, Gag and Pol and that the precursor frequency of HIV-1-specific CTL is unusually high in such individuals (Hoffenbach et al., 1989; Gotch et al., 1990; Carmichael et al., 1993). On the other hand it has been postulated that excess CTL activity may contribute to the immunopathogenesis of AIDS (Zinkernagel & Hengartner, 1994; Koenig et al., 1995).

CTL in HIV-2 infection has not been investigated as intensely as in HIV-1 infection. HIV-2-infected individuals live longer than HIV-1-infected individuals (Chapter 1). Thus research into the immune mechanisms which account for these differences may provide a more general insight into the pathogenesis of HIV disease. Therefore this chapter has investigated CTL activities against various HIV-2 viral proteins at different stages of HIV-2 and HIV-1/HIV-2 dual infection using autologous target cells and related them to HIV-2 proviral load.

5.b. Results

i. Study subjects

Twenty HIV-2 seropositive subjects and 4 subjects seropositive for both HIV-1 and HIV-2 were recruited from the hospital-based study in The Gambia. Their clinical status is shown in Table 5.1. None of the study subjects were suffering from an acute disease at the time of evaluation except for subject F19 who had fever, which was not due to malaria or bacteraemia. All subjects were tested for CTL between 20th January and 8th April 1994 during the dry-season when clinical malaria in adults is rare.

ii. HIV-2 specific CTL activities

CTL responses against HIV-2 Gag, Pol and Nef protein in 16 HIV-2 seropositive, and 4 HIV-1/HIV-2 dually seropositive individuals after day-14 of culture are shown in Table 5.1. A CTL response with a HIV-specific lysis of ≥10% to Gag or Pol protein was seen in 18/20(90.0%) or 14/20(70.0%) of the subjects respectively whereas a CTL response against Nef protein was apparent in only 5/20 (25.0%) individuals. Fifteen subjects had a CD4% of ≥ 14%. All of these subjects recognised at least one HIV protein with a HIV-specific lysis of ≥ 10%; 10 (66.6%) showed a HIV-specific lysis of ≥ 10% at two E:T ratios. Fifteen of the subjects were asymptomatic. All asymptomatic subjects regardless of their CD4%, responded to at least one of the HIV proteins; 13 (86.6%) showed a HIV-specific lysis of ≥ 10% at two E:T ratios. In 14/20(70.0%) subjects multiple HIV proteins, usually Gag and Pol, were simultaneously recognised.

Table 5.1.
HIV-2-specific lysis at various E:T ratios to Gag, Pol and Nef proteins and its relation to CD4%, proviral load and clinical status.

Subject	#HIV-specific lysis (%) against									CD4 (%)	βProviral load	Clinical status
	Gag			Pol			Nef					
F21	**38**	**45**	**19**	7	0	3	**8**	0	0	38	3.4	Asymptomatic
F12	**46**	**29**	**19**	**27**	**13**	**14**	4	2	0	39	3.6	Asymptomatic
F16[α]	4	5	3	**21**	**11**	0	**14**	0	**14**	35	4.9	Asymptomatic
F38	**27**	**11**	**15**	**26**	**24**	**15**	3	2	1	20	8.9	Asymptomatic
F22	**33**	**18**	9	**17**	**11**	1	6	0	0	38	14	Asymptomatic
F24	**19**	**18**	4	9	7	3	0	0	0	16	15	Asymptomatic
F17*	**18**	**36**	**24**	**19**	**23**	9	5	6	**11**	20	18	Asymptomatic
F2	**16**	**12**	3	**20**	**12**	0	2	0	0	13	54	Asymptomatic
F5*	**32**	**13**	6	**22**	**13**	**13**	**11**	4	0	26	115	Asymptomatic
F6	**36**	**17**	**11**	**21**	**18**	**17**	**11**	**13**	4	30	157	Asymptomatic
F14	**21**	**18**	**13**	**19**	**26**	**11**	0	0	0	25	223	Asymptomatic
F39	**14**	8	1	**18**	7	0	4	0	0	9	472	Asymptomatic

Table 5.1. continued.

F32	**19**	2	0	0	0	0	0	0	0	30	700	Symptomatic
F4	**37**	**24**	**15**	**16**	**12**	**17**	9	2	5	27	897	Asymptomatic
F18*	**18**	9	4	7	4	0	9	9	6	34	1053	Asymptomatic
F25*	**16**	4	6	**13**	5	7	3	0	1	23	1497	Symptomatic
F20	**26**	7	3	**14**	1	0	7	2	2	6	2307	Symptomatic
F3	**29**	**18**	6	2	2	0	8	2	0	30	3478	Asymptomatic
F11	**11**	6	2	**11**	6	0	**10**	0	0	7	36674	Symptomatic
F19	0	0	0	2	0	0	4	0	0	6	69345	Symptomatic

[#]The numbers indicate HIV-specific lysis (%) at 60:1, 30:1 and 15:1 E:T ratio from the left to right; HIV-specific lysis of 10% or greater is indicated in bold letters.

[*] HIV-1 and HIV-2 dually seropositive individuals.

[α]A high background of ^{51}Cr release (35-48%) was noticed in this experiment.

[β]Number of HIV-2 proviral copies / 10^5 $CD4^+$ cells are shown.

iii. Comparison of CTL activities and HIV-2 proviral load

In order to examine the relationship between HIV-2 specific CTL activity and HIV-2 proviral load, HIV-specific lysis (%) of day-14 culture at an E:T ratio of 30:1 was taken as a representative quantitative value. The coefficients of correlation between HIV-specific lysis to each HIV-2 gene and the HIV-2 proviral load expressed as copies/10^5 $CD4^+$ cells among 16 HIV-2 seropositive subjects and 4 HIV-1/HIV-2 dually seropositive subjects are shown in Table 5.2. Significant relationships were found between viral load and Gag-specific CTL or, to a less extent, Pol-specific CTL ($p<0.05$), but not to Nef-specific CTL. Total HIV-2-specific CTL activity was calculated as the sum of HIV-specific lysis (%) against three target genes: a strong relationship between the sum of HIV-specific lysis and the viral load was found (Table 5.2, Figure 5.1.A). In 11/19 (57.9%) of subjects who significantly recognised HIV-2 proteins, the highest HIV-specific lysis was against Gag protein. In 7/19 (43.8%) of subjects, the highest HIV-specific lysis was against Pol protein and in 1/19 (5.3%) against Nef protein. When the highest specific lysis was regarded as dominant CTL activity, there were even stronger relationships found between HIV-specific lysis of the dominant CTL and viral load (Table 5.2, Figure 5.1.B). A weak but non-significant correlation between CD4% and dominant CTL activity was noted (Table 5.2). There was no significant relation found between $CD4^+$ cell count, CD8% or $CD8^+$ cell count and HIV-specific lysis (data not shown).

Specific lysis (%) to		Gag	Pol	Nef	all 3 targets	dominant target
Viral load (log DNA copies/10^5CD4+)	r	-0.596	-0.456	-0.027	-0.625	-0.674
	p	0.006	0.043	0.910	0.003	0.001
CD4%	r	0.444	-0.004	0.204	0.340	0.398
	p	0.050	0.986	0.388	0.143	0.083

Table 5.2.
Correlation between HIV-2-specific lysis to different gene products and HIV-2 proviral load or CD4%.

The coefficients of correlation (top) and p value (bottom) are shown.

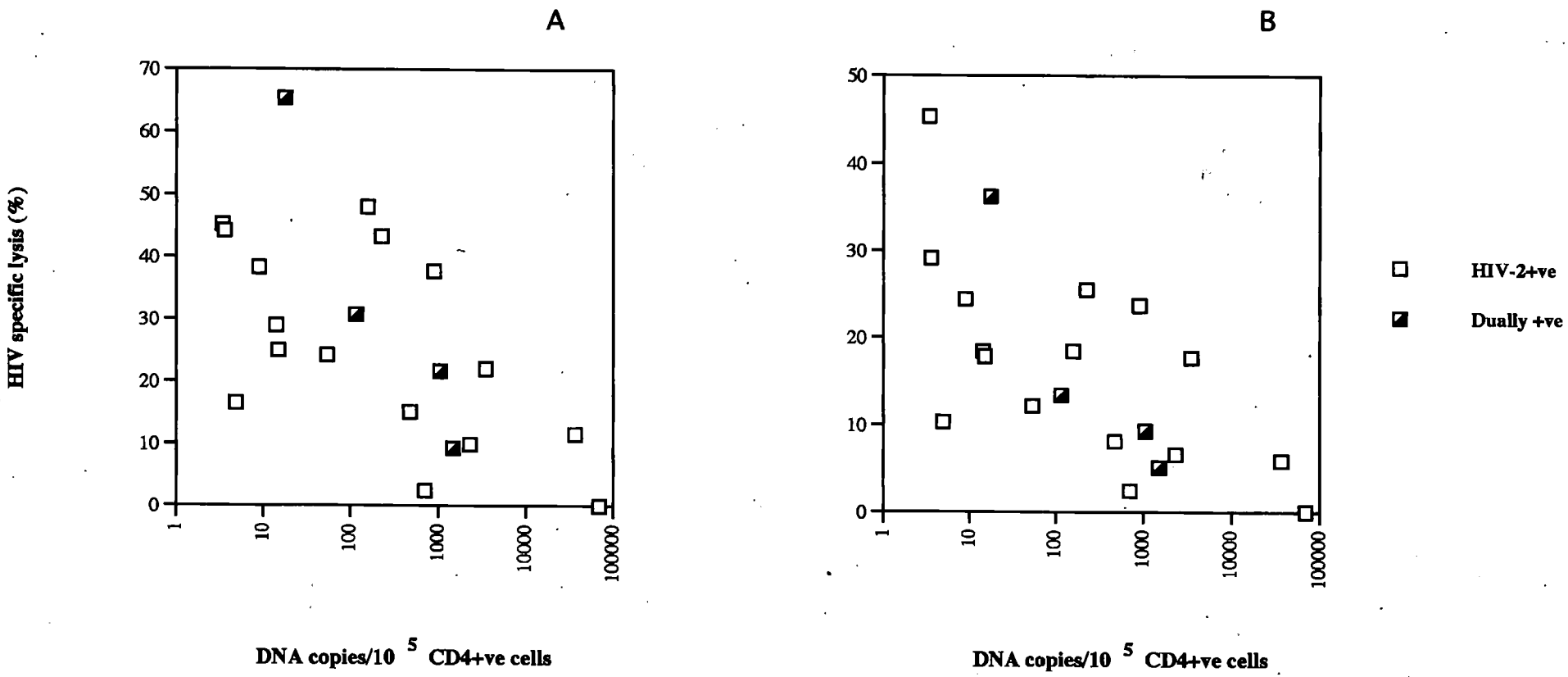

Figure 5.1.
Correlation between HIV-2 proviral load and HIV-2-specific CTL activities.
HIV-2 proviral load is compared with the sum of HIV-specific lysis at 30:1 E:T ratio (A) or HIV-specific lysis at 30:1 E:T ratio of the dominant CTL response (B).

5.c. Discussion

HIV specific CTL may be presented by multiple class I HLA molecules (McMichael & Walker, 1994). Thus in order to analyse the total CTL activity, autologous target cells were utilised in our assay.

Significant HIV-2-specific CTL against Gag and/or Pol proteins was demonstrated in the majority of the study subjects especially if they had a CD4% ≥14% or if they were asymptomatic. Similar observations have been made in HIV-1 infected asymptomatic individuals (Walker et al., 1988; Nixon et al., 1988; Lamhamedi-Cherradi et al., 1992). The demonstration of significant HIV-2-specific CTL in most of HIV-2-infected individuals suggests that the better prognosis of HIV-2-infected individuals is not due to absent or low CTL activity with consequent sparing of CD4+ cell destruction, as suggested in the hypothesis of Zinkernagel RM. and Hengartner H.(Zinkernagel & Hengartner, 1994).

It was also noticed that in many subjects multiple HIV proteins were simultaneously recognised. Whether CTL against one target protein is sufficient to suppress viral replication or whether CTL against multiple HIV proteins work synergistically to control the virus replication remains unknown. Individual target-specific CTL activity did not have as strong a relation to viral load as the sum of CTL activity against three targets. However it is noteworthy that when the dominant CTL activity was plotted against viral load the best inverse correlation was seen. This observation suggests that the dominant CTL against any one of target proteins may be particularly important for suppressing viral replication. The infrequent recognition of Nef by CTL in our study subjects may have been due to sequence variation between the Nef gene in the recombinant virus, which was derived from HIV-2_{BEN} and Nef gene in the wild-type virus prevailing the Gambia. The wild-type virus may relate more closely to HIV-

2_{ROD} from which *gag* and *pol* target genes were originated. Alternatively infrequent recognition of Nef by CTL may be characteristic of HIV-2 infection.

The use of specific lysis in bulk-culture to quantitate CTL activity is generally considered to be less accurate than limiting dilution analysis(LDA) (Carmichael et al., 1993). However Gallimore et al. have recently shown a clear correlation between precursor frequency determined by LDA and specific lysis by a bulk culture after 14 days at 30:1 E:T ratio (Gallimore et al., 1995). Also the LDA method suffers from a number of problems, in particular how to arbitrarily define the cut off between positive and negative responses, and how to deal with nonspecific background lysis. In order to standardise this test, the conditions of the bulk culture, the day when the bulk culture was tested and the E:T ratio were strictly controlled. The data was also analysed using the specific lysis at 15:1 or 60:1 or the mean specific lysis of three E:T ratios to express CTL activity quantitatively. Whichever value was used, a significant relation between the HIV-specific lysis and viral load was seen ($p<0.05$). Although HIV-specific lysis was lower in day-7 cultures, this measurement was also significantly and inversely correlated to proviral load (data not shown). Thus this data provides evidence to support the hypothesis that HIV-2-specific CTL play an important role in controlling HIV-2 replication.

Proviral load, which is a direct measure of the number of both latent and productive infection in cells, seems relatively slow to change (Perelson et al., 1997). On the other hand plasma viral load appears to be an index of the number of cells producing viruses; the turnover of productively infected cells, which is very rapid, might be responsible for the pathogenesis of AIDS (Chapter 1). It would be interesting to relate plasma viral load to CTL activity, since CTL are likely to be directed primarily at cells actively producing viruses. Nonetheless in this study a clear relationship was

observed between CTL activity and proviral load, implying that the virus-CTL relationship in HIV-2 infection may have reached a steady state where the dominant CTL may be directed against conserved regions of the virus (Nowak & Bangham, 1996). In HIV-1 infection, one study found that there was an inverse relationship between HIV-1 Env-specific CTL activity and proviral load (Kundu & Merigan, 1994) but another study did not find such a correlation (Rinaldo et al., 1995b). The virus-CTL relationship in HIV-1 infection may differ where the dominant CTL may target more variable regions of the virus.

There was one subjects (F16) who had low viral load of less than 10 copies/10^5CD4$^+$ cells but had relatively low CTL activities. The first CTL results of subject F16 may not be accurate because of a high background of ^{51}Cr release (≥35%). However in the second experiment, 2 years after the first experiment, the subject was found to have a very strong CTL activity against Gag (Figure 5.2). It was also noticed that there were several subjects {F3, F4, F6, F14} with HIV-2 single infection who had high viral load of >100 copies/10^5CD4$^+$ despite high CTL activity of significant lysis at least two E:T ratios. As previously argued in HIV-1 infection (Phillips et al., 1991), mutant viruses may have emerged in the subject and escaped the CTL check.

5.e. Longitudinal observation of HIV-2-specific CTL.

Although the first part of this chapter demonstrated a gross correlation between a proviral load and the CTL activities, the study subjects were a mix of early stage and late stage of either HIV-2 single or HIV-1/HIV-2 dual infection. Of interest was to compare HIV-2-specific CTL activities longitudinally among HIV-2 singly infected individuals with a relatively high CD4% (≥20%) particularly between those with a low proviral load (<15 copies / 10^5 CD4$^+$ cells) and with a high proviral load (>100 copies / 10^5 CD4$^+$ cells). CTL activities have been repeatedly measured in 5 subjects

with a low proviral load {F21, F12, F22, F16, F38} and 3 subjects with a high proviral load {F6, F14, F3}, over the following two years. As Gag CTL dominated among these subjects, HIV-2-Gag specific CTL activities during the three years of follow-up are shown in Figure 5.2. Subject F4 was excluded from the analysis as the subject was also infected with HIV-1. The 5 subjects with a low proviral load were found to have a strong CTL throughout the period of observation whereas the 3 subjects with a high proviral load despite a singificant level of CTL in first year of the study, were found to have a low level of CTL activities in third year of the study. These observations suggest that the virus-CTL relationship is stable among HIV-2-infected long-term non-progressors where a high level of CTL is maintained perhaps by a low level of viral antigen presentation. On the other hand, among progressors the virus-CTL relationship is not stable as CTL activities tend to fluctuate. Interestingly in such progressors an increase in viral load precedes the decline of CTL activities. As it was shown in animal experiments, over-presentation of viral antigen may have induced anergy (Rocha et al., 1993). Alternatively a high rate of viral replication may have impaired $CD4^+$ T cell function which is required for maintenance of CTL activities, as it was reported that cytomegalovirus-specific helper cells are needed to maintain cytomegalovirus-specific CTLs (Walter et al., 1995).

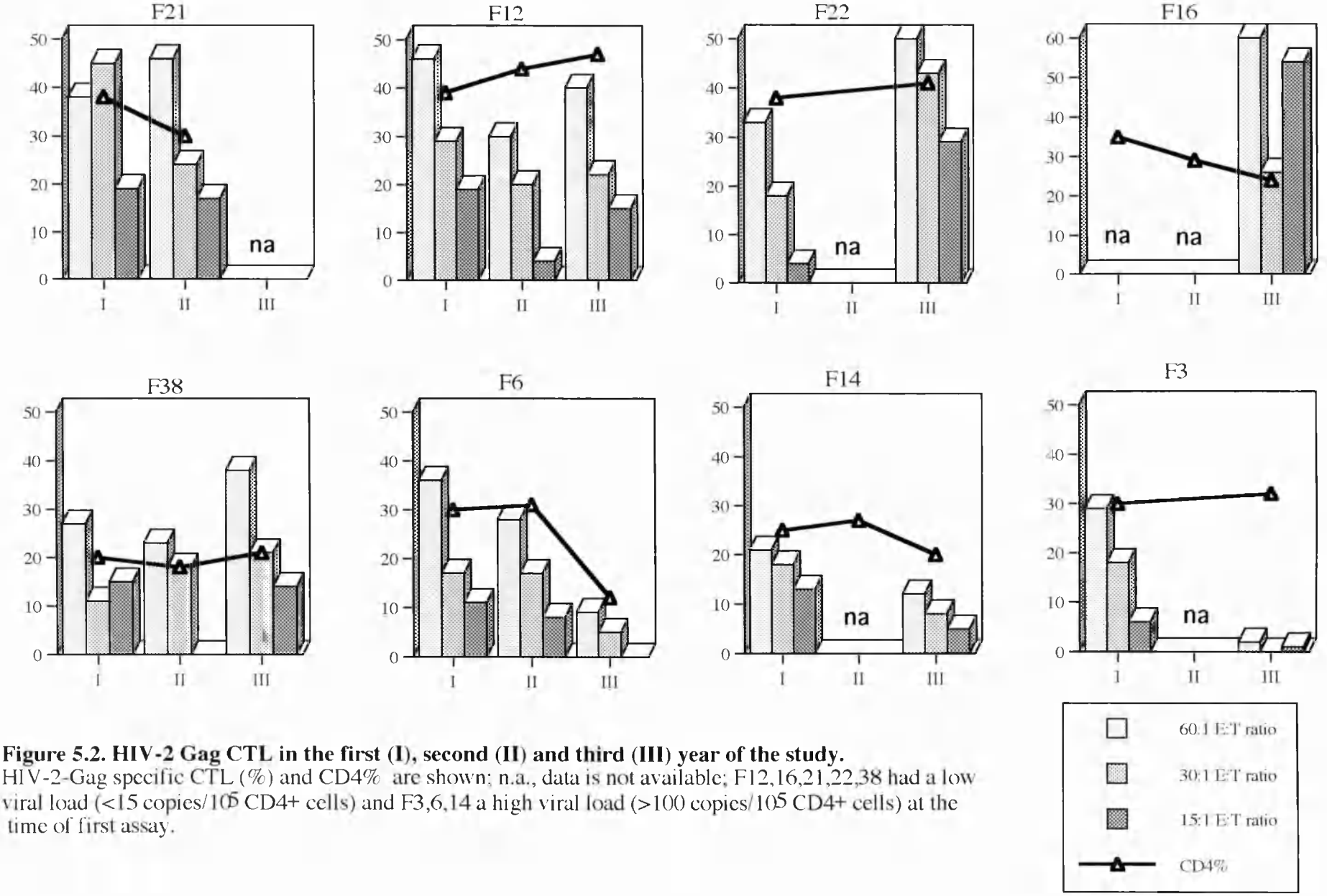

Figure 5.2. HIV-2 Gag CTL in the first (I), second (II) and third (III) year of the study.
HIV-2-Gag specific CTL (%) and CD4% are shown; n.a., data is not available; F12,16,21,22,38 had a low viral load (<15 copies/10^5 CD4+ cells) and F3,6,14 a high viral load (>100 copies/10^5 CD4+ cells) at the time of first assay.

Chapter 6.

Effects of various co-infections on HIV-2 viral load

Many infectious diseases are more common in Africa than in the developed countries. Thus HIV-infected individuals living in Africa are more likely to be exposed to and co-infected with other infectious diseases. Several co-infections and immunogens such as influenza virus (Ho et al., 1992), herpes simplex virus-1 (Heng et al., 1994), *Mycobacterium tuberculosis* (Goletti et al., 1996), and bacterial pneumonia (Bush et al., 1996) and vaccination (Staprans et al., 1995; Stanley et al., 1996) have been shown to increase HIV-1 replication. In this chapter, the effect of HTLV-I and malaria has been studied.

6.a. Lack of *in vivo* evidence for the enhancement of HIV-2 infection by HTLV-I.

6.a.1. Introduction.

Both Human Immunodeficiency Virus Type-2 (HIV-2) and Human T-Lymphotrophic Virus Type-I (HTLV-I) are endemic in West Africa (Pepin et al., 1991a; Naucler et al., 1992; Biggar et al., 1993; Dada et al., 1993; Hishida et al., 1994; Norrgren et al., 1995; Chapter 3). The viruses are transmitted horizontally through sexual intercourse or contaminated blood or vertically from mother to child and both establish a persistent infection (reviewed by Centers for Disease Control and Prevention, 1993). Risk factors for infection are shared by the two retroviruses, consequently individuals who harbour one virus, may often be co-infected with the other virus (Pepin et al,, 1991a; Naucler et al., 1992; Bigger et al., 1993; Norrgren et al., 1995). At the cellular level, both viruses infect $CD4^+$ lymphocytes thus there may be co-infection of the same host cell *in vivo* (Richardson et al., 1990). In vitro experiments have suggested several direct and indirect mechanisms by which HTLV-I may augment the level of HIV-1 replication although only few studies have been performed for HIV-2 (Zack et al., 1988; Lusso et al., 1990; Sakai et al., 1990; Lewis et al., 1990; Landau et al.,

1991; McGuire et al., 1993). Evidence is accumulating to show that increased HIV-1 replication is responsible for CD4 decline (Chapter 1). Thus co-infection with HTLV-I has also been thought to enhance the rate of HIV disease progression by increasing HIV virus replication. Nonetheless little is known about HIV virus load among those infected with both HIV and HTLV-I and despite several clinical studies, it is still controversial whether HTLV-I co-infection influences the course of HIV disease (Page et al., 1990; Murakami et al., 1991; De Rossi et al., 1991; Visconti et al., 1993; Schechter et al., 1994).

The overall genetic diversity of HTLV-I within a carrier is less than that of HIV-1 or HIV-2 (Gessain et al., 1992; Wattel et al., 1995). However the level of HTLV-I provirus has been found to vary remarkably being up to 100,000 fold different between infected individuals (Gessain et al., 1991; Matsumura et al., 1993). This variation in the level of HTLV-I provirus in carriers may be important in the pathogenesis of HTLV-I infection and its interaction with HIV disease. HIV-2 proviral load has been investigated in a rural village of Guinea-Bissau where HIV-2 is endemic and a high prevalence of HTLV-I infection was also found (Chapter 3). However the study did not show a significant difference in the geometric mean HIV-2 provirus load between those with and without HTLV-I infection. It was postulated that the effect of HTLV-I co-infection on HIV disease or HIV replication may vary according to the level of HTLV-I virus load. Thus in this chapter HTLV-I provirus load was determined in these villagers and was related to HIV-2 provirus load, and conversely assessed the effect of HIV-2 infection on HTLV-I provirus load.

6.a.2. Results

i. Study subjects

As described in Chapter 3, 132 HIV-2 seropositive subjects and 160 HIV-seronegative subjects were recruited in a rural village in Guinea-Bissau between January and May 1991 of which PBMC samples were available from 127 HIV-2 seropositive subjects and 160 HIV-seronegative subjects. HTLV provirus DNA was detected in 31 (24.4%) of the HIV-2 seropositive PBMC samples and in 15 (9.4%) of the HIV seronegative samples by qualitative PCR (Chapter 3). All HTLV PCR positive samples were diagnosed as HTLV type I by the restriction enzyme analysis.

ii. Characteristics of subjects

The mean age, sex ratio, mean CD4% of subjects in the HIV-2-seropositive and HIV-seronegative subjects with or without HTLV-I infection are shown in Table 6.1. In HIV-2-seropositive subjects, HTLV-I infection was significantly associated with older age ($p = 0.01$, t test) and with being female ($p < 0.0001$, Fisher's exact test) and the mean CD4% was significantly higher in HTLV-I infected individuals than in HTLV-I uninfected individuals ($p = 0.049$, t-test). However these associations were not significant in the HIV-seronegative subjects.

	HIV-2 seropositive		HIV-2 seronegative	
HTLV-I status	positive (n=31)	Negative (n=96)	positive (n=15)	negative (n=145)
Proportion female	30/31 (96.8%)	58/96 (60.4%)	11/15 (73.3%)	108/145 (74.5%)
Mean±S.E age (years)	56.1±2.8	48.5±1.4	54.3±4.6	49.9±1.4
mean±S.E. CD4 %†	32.9±1.7	28.6±1.1	36.5±1.6	35.5±0.7

Table 6.1.
The sex ratio, mean age and mean CD4% in subjects according to HTLV-I and HIV-2 infection.
† CD4% was available from 110 HIV-2 seropositive and 141 HIV seronegative subjects.

iii. HTLV-I viral load among HIV-2 positive individuals and HIV negative individuals

Figure 6.1 shows the distribution of HTLV-I provirus copies per 1μg of DNA in HIV-2 seropositive individuals and HIV negative individuals. The median value for the HIV-2-infected subjects was 3125 (Interquartile range (I.R.), 625, 3125) copies / 1 μg DNA which was higher than the median of the control group which was 625 (I.R., 25, 15625) copies / 1 μg DNA but the difference was not statistically significant ($p = 0.6$, Wilcoxon test). On the assumption that the $CD4^+$ cell fraction harboured most HTLV-I virus (Richardson et al., 1990), the number of provirus copies per $CD4^+$ cell fraction was calculated by dividing the number of provirus copies per 1 μg PBMC DNA by CD4%. There were 25 HTLV-I/HIV-2 dually infected subjects and 15 HTLV-I singly infected subjects for whom valid CD4% data was available. The median number of HTLV-I provirus copies / $CD4^+$ cell fraction in the dually infected subjects was 3289 (I.R. 1389, 15625) which was not significantly different from the median of 1953 (I.R. 60, 33245) observed in the singly infected group ($p = 0.7$, Wilcoxon test). The number of provirus copies / 1 μg PBMC DNA was not significantly related to CD4% in 25 dually infected subjects with a valid CD4% data ($r = - 0.049$, $p = 0.8$ Spearman's test), nor did HTLV-I provirus level relate to age ($r = -0.07$, $p = 0.7$, Spearman's test).

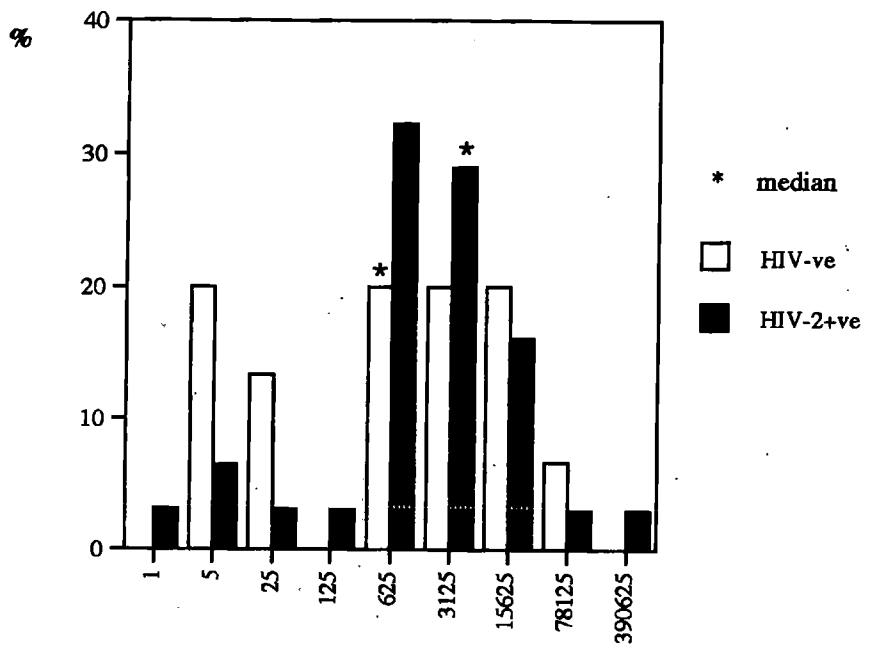

Figure 6.1.
Histogram of HTLV-I proviral load in HIV-2-infected and HIV-uninfected subjects

Subject No.	Type of Dilution	Dilution of cells or DNA				
		1/ 3125	1/ 15625	1/ 78125	1/ 390625	1/ 1953125
c61	Cell	NT	+ 2/2	**+ 4/4**	- 0/4	- 0/4
	DNA	NT	+ 1/1	+ 4/4	**+ 3/4**	- 0/4
c135	Cell	+ 4/4	**+ 2/4**	- 1/4	NT	NT
	DNA	+ 4/4	+ 4/4	**+ 2/4**	- 0/4	NT

Table 6.2 Comparison of limiting dilution analysis using two different methods of sample dilution.
It shows the number of positive reactions / number of reactions tested at a dilution of 1.5×10^5 cells or equivalent amont of celluar DNA; NT, not tested; the end-point is indicated in bold lettering; a cellular gene, β-globin gene was detected by nested PCR at 1/78125 dilution but not at 1/390625 dilution in both samples prepared by two different methods.

Table 6.2 shows results of limiting dilution analysis of either PBMC or DNA from the two subjects, c61 and c135, who had the highest level of HTLV-I proviral load among HIV-2 seropositive and HIV-seronegative subjects respectively. In subject c61 who was a 66 years old female with a CD4% of 35, the end-point dilution was 1/390,625 when DNA sample was diluted whereas it was 1/78,125 when the cell sample was diluted before DNA extraction. Similarly in subject c135 who was a 65 years old male with a CD4% of 28, the end-point dilution was 1/78,125 when DNA was diluted, while it was 1/15,625 when cells were diluted. The dilution experiments were repeated and a consistent result was obtained. Thus in both cases the end-point was about five-fold higher if DNA was extracted before diluting sample than after diluting sample.

iv. Comparison between HTLV-I viral load and HIV-2 proviral load

HTLV-I provirus level was plotted in relation to HIV-2 provirus level which was determined in Chapter 3, in the 31 dually infected individuals (Figure 6.2). There was no positive correlation between provirus level of HTLV-I and HIV-2 before ($r = -0.33$, $p = 0.073$, Spearman's test) or after adjusting for CD4% ($r = -0.32$ $p = 0.12$, Spearman's test).

After 4 years of follow-up, 4 among 31 (12.9%) HIV-2/HTLV-I dually infected subjects, 11 out of 96 (11.5%) HIV-2 singly infected subjects and none of the 15 HTLV-I singly infected subjects had died. There were 13 deaths among the 145 (9.3%) HIV/HTLV-I uninfected subjects. After adjusting for sex and age in the HIV-2 infected group, the risk of death from HTLV-I infected subjects relative to HTLV-I uninfected subjects was 1.32 (95% C.I. 0.31, 5.56) ($p = 0.7$). The overall risk of death from HTLV-I infection adjusted for age, sex and HIV status was 0.66 (95% C.I. 0.20, 2.19) ($p=0.5$).

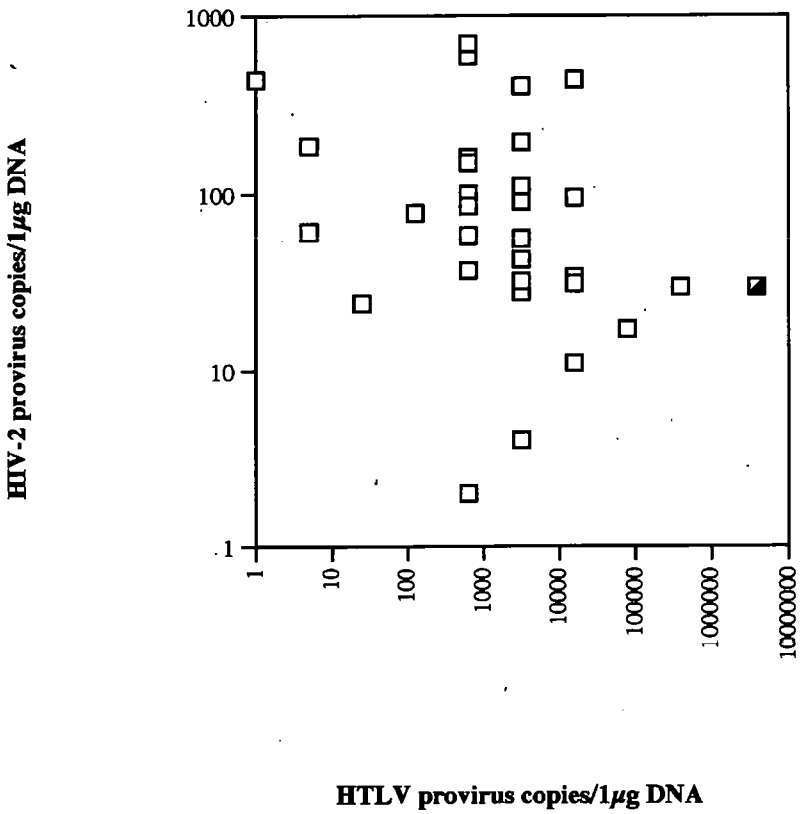

Figure 6.2. Relation of HTLV-I proviral load to HIV-2 proviral load. Subject (c61) who had a very high HTLV-I viral load is indicated by a shaded square.

6.a.3. Discussion

In this HIV-2 seropositive population, HTLV-I infection was associated with age and was seen more frequently in females which is consistent with previous findings in West Africa (Naucler et al., 1992; Biggar et al., 1993). This data also supports the previous finding that there is a higher CD4% in HTLV-I/HIV dually infected individuals than in HIV singly infected individuals (Schechter et al., 1994).

HTLV-I proviral load in the villagers was found to vary considerably by more than 100,000 fold. A surprisingly high level of HTLV-I provirus was seen in three subjects; two HIV-2-infected and one HIV-uninfected. Two of these subjects were estimated to have HTLV-I provirus load of 0.8×10^5 copies and one subject had 3.9×10^5 copies / 1 μg DNA (Table 6.2). Assuming that 1μg of DNA is equivalent to 1.5×10^5 cells and that $CD4^+$ cells which constitute approximately 30% of PBMCs are the only cell fraction harboring HTLV-I (Richardson et al., 1990), the two subjects were estimated to have had 1.7×10^5 copies and the other one had 8.7×10^5 copies per 1.0×10^5 $CD4^+$ cells. Thus there were more HTLV-I proviruses than the number of susceptible cells. When DNA was extracted after diluting the PBMCs in two of the subjects: c61 and c135 (Table 6.2), it was found that the number of infected cells was at least five-fold less than the number of HTLV-I provirus, suggesting that an infected cell contains on average five copies of HTLV-I provirus. Assuming that the number of infected cells was five-fold less than the number of HTLV-I proviruses, subject c135 seemed to have had more than 30% of $CD4^+$ cells infected with the virus whereas subject c61 appeared to have had almost all $CD4^+$ cells infected.

Several mechanisms by which HTLV-I could stimulate HIV replication have been tested *in vitro*. The HIV-2 *rev*-responsive element has been reported to be responsive to the HTLV-I *rex* gene product (Lewis et al., 1990; Sakai et al., 1990) and HTLV-I

tax and *rex* products interact indirectly with the LTR region by up-regulating the expression of some cellular factors such as IL-2 and IL-2 receptors (McGuire et al., 1993). In addition, pseudotype virus formation which confers on HIV-1 a wider cell tropism, is thought to be another mechanism (Lusso et al., 1990; Landau et al., 1991). Whatever mechanisms are involved, the expression of HTLV-I genes is crucial for the interaction with HIV; hence the effect of HTLV-I on HIV replication *in vivo* is thought to relate to the level of HTLV-I provirus. However in this study any positive relation between HTLV-I proviral load and HIV-2 proviral load was not found. Infection of both HIV-2 and HTLV-I within a single cell may be required to produce a synergistic effect and to significantly influence the basal level of transcription of HIV-2. It is of interest to note that one subject (c61) who had a saturated level of HTLV-I provirus and in whom many $CD4^+$ cells were likely to have been doubly infected, did not have a high HIV-2 proviral load (Figure 6.2). Final conclusions should not be drawn from the HIV-2 proviral load data but will have to wait until the HIV-2 plasma RNA viral load data is available. However the measurement of HIV proviral load is probably sensitive enough to detect the effect of HTLV-I since the HTLV-I establishes persistent infection which is thought to influence HIV-2 replication chronically. Thus the above evidences of viral load of HTLV-I and HIV-2 in doubly infected individuals suggest that HTLV-I infection may not have a marked effect on HIV-2 replication *in vivo* and therefore may not affect HIV-2 mortality. So far four years follow-up data support this concept for the mortality rate among HIV-2-infected individuals with HTLV-I co-infection was not higher than that of those without HTLV-I co-infection. However because of the long incubation time of HIV-2 disease, formal determination of effect by HTLV-I on HIV-2 disease progression may require a much longer follow-up.

HTLV-I has been known to cause various clinical manifestations such as adult T-cell

leukemia and lymphoma, tropical spastic paraparesis/HTLV-I-associated myelopathy, uveitis, arthropathy, broncopeumonitis, dermatitis (reviewed by CDC, 1993). The pathogenesis of such diseases remains poorly understood. However if any of the HTLV-I associated diseases is directly affected by the burden of virus (Gessain et al., 1991), it is important to know whether HIV infection influences HTLV-I viral load or not. In this study it could not be demonstrated that HIV-2 infection influenced on HTLV-I proviral load though the number of subjects may not have been large enough to demonstrate a small effect. A clinical survey of HTLV-I-associated diseases in the village is currently in progress to address some of these issues.

6.b. The impact of *Plasmodium falciparum* parasitaemia on HIV infection

6.b.1. Introduction

Malaria infection can be prevented by affordable prophylactic drugs in many developing countries where both HIV and malaria infections are common. In West Africa, many HIV-infected individuals are living in a malaria hyper-endemic area. Therefore it is important to ascertain if infection with *P. falciparum* affects HIV disease progression and transmission. Although the effect of HIV infection on malaria has been studied by a number of groups (Simooya et al., 1988; Muller et al., 1990; Allen et al., 1991; Greenberg et al., 1991), little is known of the impact of malaria on HIV infection. In Chapter 3, it was shown that the geometric mean HIV-2 proviral load was not different between individuals with and without *P.f.* parasitaemia. However since malaria parasitaemia is thought to be transient and because most villagers have intermittent parasitaemia, it would be difficult to demonstrate the effect of malaria by cross-sectional study. Therefore in this chapter, viral load of HIV-infected adults living in a rural village in Guinea-Bissau were compared in the end of the dry-season, when there is little transmission of malaria parasites, and in the following wet season when malaria infection is common.

6.b.2 Results

i. Study subjects

A total of 22 HIV-uninfected and 33 HIV-infected adults of whom 2 were HIV-1 and 31 were HIV-2 positive, living in the village, were bled in May 1996 just before the first rain started and in October 1996 at the peak of the rains when number of malaria cases is usually high.

ii. Prevalence and density of Plasmodium falciparum parasitaemia

Overall the prevalence of *P.falciparum* parasitaemia increased slightly during the wet season from 4/55(7.3%) to 7/55 (12.7%) and the median (interquartile range) density of parasitaemia increased significantly from 17.5 (15 – 135) parasites per μl of blood to 335 (65 – 1000) parasite per μl of blood. Four HIV-infected individuals: 1 HIV-1 and 3 HIV-2 were found to have *P.falciparum* parasitaemia at the time of sampling: one during the dry and 3 during the wet season while 29 HIV-infected individuals had no detectable parasitaemia in both blood samples.

iii. HIV viral load

HIV RNA was detected in plasma samples of 25/33 (76%) adults during the dry season and 23/33 (70%) during the wet season. In 8 HIV-2 adults, HIV-2 RNA was not detected in both samples whereas in all adults HIV proviral DNA was detected in both samples. Geometric means (95% confidence interval) of HIV RNA viral load among adults in whom HIV RNA was detected in either samples and of DNA viral load were summarised in Table 6.3.

Overall there is no tendency of increase in either HIV RNA or DNA viral load during the wet season as compared to during the dry season. Interestingly among adults without *P.f.* parasitaemia in both samples, a significant decline of RNA viral load was

noticed during the wet season. On the contrary, there was a tendency for both RNA and DNA viral load to increase among adults at the time they had *P.f.* parasitaemia (Figure 6.3). This increase in RNA viral load among adults with *P.f.* parasitaemia was significant as compared with the change observed among adults without *P.f.* parasitaemia ($p=0.015$ by t-test). However if RNA viral load was adjusted for season, the difference was not significant ($p=0.11$). The increase in DNA viral load among adults with *P.f.* parasitaemia was not significant ($p=0.1$ by t-test).

	Dry season	Wet season	Difference*
RNA viral load in all subjects (n=25)	4074 (1585, 10233)	2570 (1096, 6166)	p=0.3
RNA viral load in subjects without *P.f.* parasitaemia (n=21)	4786 (1660, 13804)	2884 (1096, 7762)	P=0.035
DNA viral load in all subjects (n=27)	7.2 (3.2, 17.0)	9.1 (3.7, 22.4)	p=0.3
DNA viral load in subjects without *P.f.* parasitaemia (n=21)	7.6 (3.1, 19)	9.1 (3.4, 24.5)	P=0.7

Table 6.3.
Geometric means (95% C.I.) of RNA viral load and DNA viral load between the dry season and the wet season.
Geometric means of RNA copies / ml of plasma and DNA copies / 10^5 PBMC are calculated; adults without detectable RNA were excluded from calculation for geometric RNA viral load.
*The significance of difference was tested by paired t-test.

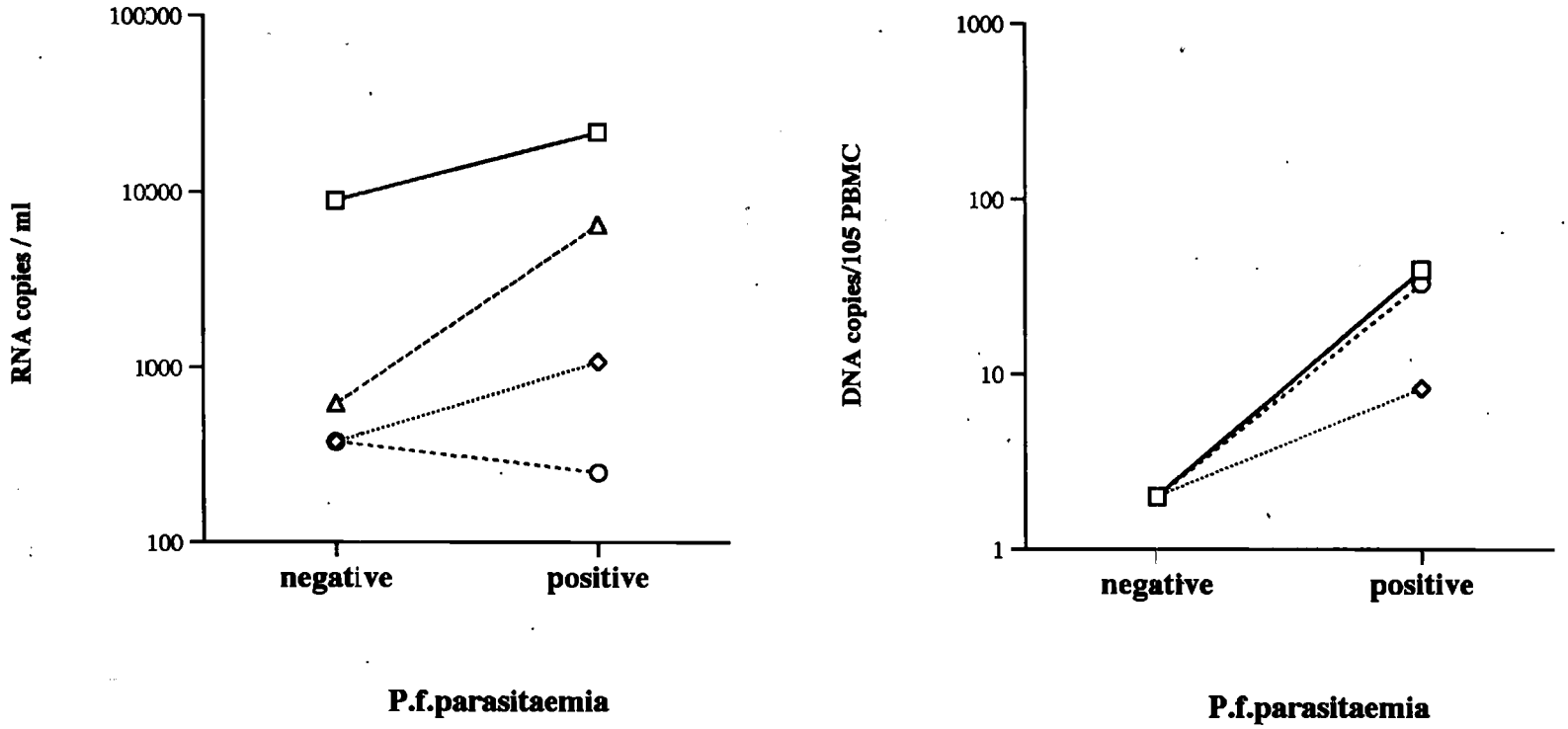

Figure 6.3. RNA and DNA viral load in samples from 4 patients with and without P.f. parasitaemia.

iv. Changes in $CD4^+$ cell count

CD4/CD8 subset data was available for 32 HIV-positive adults and 23 HIV-negative controls. Changes in CD4 and CD8 count are summarised in Table 6.4. Although CD4% was stable between the dry season and the wet season among HIV-positive adults, a significant decline of absolute CD4 count was observed during the wet season. Among HIV-negative controls, CD4% was significantly higher during the wet season but the absolute CD4 count was not significantly different between the seasons. In both HIV-positive and negative groups, a significant decline of lymphocyte count was noticed. However no significant change of CD4 count was associated with parasitaemia in either group.

Group		Dry season	Wet season	Difference
All HIV-positive adults (n=32)	CD4% (%)	32.2 (29.0, 35.3)	33.8 (31.0, 37.2)	P=0.207
	Absolute CD4 (count/μl)	1287 (1091, 1483)	976 (808, 1144)	P=0.006
	Lymphocyte (count/μl)	4088 (3502, 4674)	2916 (2456, 3376)	P=0.001
All HIV-negative controls (n=23)	CD4% (%)	38.3 (35.1, 41.5)	44.1 (40.1, 48.1)	P<0.001
	Absolute CD4 (count/μl)	1555 (1267, 1843)	1271 (1061, 1481)	P=0.09
	Lymphocyte (count/μl)	4187 (3351, 5023)	3091 (2461, 3721)	P=0.017

Table 6.4. Mean (95% C.I.) $CD4^+$ cell count in the dry season and in the wet season.

6.b.3. Discussion.

In this village, a marginal increase in the prevalence of parasitaemia but a significant increase in the density of parasitaemia was observed during the wet season. This pattern of parasitaemia in adults is typical of areas with seasonal but reasonably high levels of malaria transmission (Molineaux et al., 1988). Therefore one can expect that the population of this village is exposed to several infectious mosquito bites per annum, and that the vast majority of these occur during the wet season. Thus this preliminary observation suggests that exposure to malaria during the wet season itself does not enhance the rate of HIV replication in malaria-immune adults but the presence of detectable levels of parasites in the peripheral blood may have an enhancing effect. Malaria has been known to be a strong stimulator of B cells (McGregor et al., 1970) and induction of malaria specific immune response is likely to involve activation of T helper cells (Ballet, et al., 1987; Troye-Blombery et al., 1994). More recently blood stage antigens of *P.falciparum* have been shown to stimulate $CD4^+$ T cells of malaria-immune adults (E Lee manuscript in preparation). Increased size of activated $CD4^+$ T cells may explain the increase in HIV replication, since activated $CD4^+$ T cells are susceptible to HIV productive infection (Stevenson et al., 1990). Alternatively malaria infection may suppress HIV-specific cell-mediated immunity (Whittle et al., 1984, 1990; Riley et al., 1988). Accumulating evidences have shown that increased viral load was associated with faster rate of disease progression (Mellors et al., 1995, 1996; Henrard et al., 1995; Phillips et al., 1996; Ruiz et al., 1996; de Wolf et al., 1997). In this study, though, decline of $CD4^+$ cell count was not observed in association with parasitaemia. This was thought because in adults with partial immunity to *P.falciparum*, parasitaemia is transient and it is plausible that significant changes in $CD4^+$ cell count would only become apparent if parasitaemia persisted for longer. However even a transient increase in HIV replication may increase the probability of HIV transmission as it has been suggested

that a high maternal viral load is a strong risk factor of mother-to-child HIV-1 transmission (Fang et al., 1995; Dickover 1996). In countries where expensive anti-retroviral therapy can not be a choice of management or prevention of HIV-infection, alternative way of improving patient's life is needed.

Intriguingly among adults without *P.f.* parasitaemia, a significant decline of RNA viral load was observed during the wet season. Also a significantly higher CD4% was observed during the wet season among HIV-negative controls and a significantly lower lymphocyte count was observed in both HIV-positive and negative adults. These changes may be a reflection of the same phenomenon such as cytokine environment may change seasonally.

Chapter 7.

PCR analysis of HIV-1 and HIV-2 proviruses in dually seroreactive individuals

7.a. Comparison between qualitative PCR and serodiagnosis

7.a.1. Introduction.

As discussed in the general introduction (Chapter 1), HIV-infected individuals dually seroreactive for both HIV-1 and HIV-2 are common in West Africa but the significance of dual seroreactivity remains unclear.

Two previous studies from Abidjan in Cote d'Ivoire have shown that 41% to 62% of dually seroreactive patients, as defined by Western-Blot and specific peptide-based assays, are positive by PCR or culture for both HIV-1 and HIV-2 (George *et al*, 1992; Peeters *et al*, 1991). However a substantial proportion of these subjects remained unproven as cases of mixed infection. Several reasons for the discrepancies between PCR and serology in dually seroreactive individuals have been postulated: the sensitivity of PCR is not sufficiently high; the level of provirus is too low to be detected; cross reactive antibody mimics dual seroreactivity; patients are infected with diverse strains of either HIV-1 or HIV-2 which have a greater degree of sequence homology or with recombinant viruses of HIV-1 and HIV-2; cross-reactive immunity elicited by preceding HIV infection aborts subsequent infection by the other type of HIV.

The objectives of the first part of this chapter are to improve serological diagnosis of HIV-1/-2 dual infection and to clarify the implications of dual seroreactivity. A highly sensitive PCR assay was applied, which can detect HIV-2 provirus in over 98% of HIV-2 seropositive individuals (Berry *et al.* 1994; Chapter 3) and in 100 % of HIV-1 seropositive individuals in the region (Berry *et al.* manuscript submitted). Using this sensitive test, antibody titres in PCR singly positive subjects were compared with that of PCR dually positive subjects coming from the clinic or community.

7.a.2. Results.

i. Study subjects

From January 1991 to June 1995 patients attending the MRC hospital, who were seropositive for both HIV-1 and HIV-2 were enrolled. New dually seroreactive cases were identified by serially testing 26 HIV-1 and 53 HIV-2 seropositive CSWs. Between January 1993 and March 1995, 29,670 women attending the eight major government antenatal clinics who were part of a major study on perinatal transmission, were screened by serology for HIV (O'Donovan *et al*, 1996). Women who were dually seroreactive were also enrolled.

During the study period 78 patients at the hospital and 16 pregnant women were diagnosed as dually seroreactive based on the criteria that the sample is dually seroreactive by both competitive ELISA assays and PeptiLAV. Eight HIV-2 positive and one HIV-1 positive CSWs became dually seroreactive. Of the total 103 dually seroreactive subjects, PBMC samples were available from 76 subjects of whom 57 were hospital patients, 9 seroconverters and 10 pregnant women.

ii. Qualitative PCR versus competitive ELISA and PeptiLAV

Results of initial PCR screening of dually seroreactive subjects as identified by both competitive ELISA and PeptiLav are shown in Table 7.1. Overall 70% were found to be dually positive by PCR while 20% were positive for HIV-1 only and 9% for HIV-2 only. One subject was negative by both HIV-1 and HIV-2 by PCR. When PCR results of hospital patients, seroconverters and pregnant women were separately analysed, there was a difference in the pattern among PCR singly positive subjects: the proportion of HIV-1 PCR singly positive subjects was higher among hospital than community based subjects (15/57 vs 0/10; p=0.1); the proportion of HIV-2 PCR singly positive subjects was significantly lower among hospital-based than community-based subjects (3/57 vs 4/10; p=0.008). All seroconverters were found to be dually positive by PCR.

PCR	Hospital-based	Seroconverters	Community-based	Total
HIV-1	15 (26%)	0	0	15 (20%)
HIV-2	3 (5%)	0	4 (40%)	7 (9%)
Dual	38 (67%)	9 (100%)	6 (60%)	53 (70%)
Neg	1 (2%)	0	0	1 (1%)
Total	57 (100%)	9 (100%)	10 (100%)	76 (100%)

Table 7.1.
Results of nested PCR in subjects testing dually seropositive by PeptiLav and routine type-specific ELISA.

iii. Qualitative PCR versus HIV-1 and HIV-2 antibody titres

Anti-HIV-1 and anti-HIV-2 antibody titres could be compared in 75 dually seroreactive subjects tested by PCR (Figure 7.1.). The proportion of individuals with a low titre (<10) of antibodies was significantly different between PCR dually and singly positive subjects. None of 53 PCR dually positive subjects had a low titre of either anti-HIV-1 or anti-HIV-2 antibodies whereas 6 (40%) of 15 HIV-1 PCR positive / HIV-2 PCR negative subjects had anti-HIV-2 antibody titre of <10 ($p<0.0001$, Fisher's exact test) and 6 (86%) of 7 HIV-1 PCR negative / HIV-2 PCR positive subjects had anti-HIV-1 antibody titre of <10 ($p<0.0001$, Fisher's exact test). One subject who was negative for both HIV-1 and HIV-2 PCR and one subject who was positive for HIV-2 PCR only had a low antibody titre of <10 for both HIV-1 and HIV-2.

In order to detect HIV provirus at a very low frequency of infected cells, 22 PCR singly positive/dually seroreactive subjects were tested four times by PCR. Three of the 15 HIV-1 PCR positive / HIV-2 PCR negative subjects were found to be positive by repeated HIV-2 PCR tests. All three subjects had anti-HIV-2 antibody titre of ≥10. However no subjects with anti-HIV antibody titre <10 were positive for both HIV-1 and HIV-2 after quadruplicate PCR experiments. Based on this observation, dual seroreactivity was re-defined by the combination of a positive PeptiLav and a positive ELISA at a dilution of 1:10. 63 individuals were re-diagnosed dually seroreactive by the new definition and the final results of PCR tests are shown in Table 7.2. 89% of these dually seroreactive subjects were positive by both HIV-1 and HIV-2 PCR.

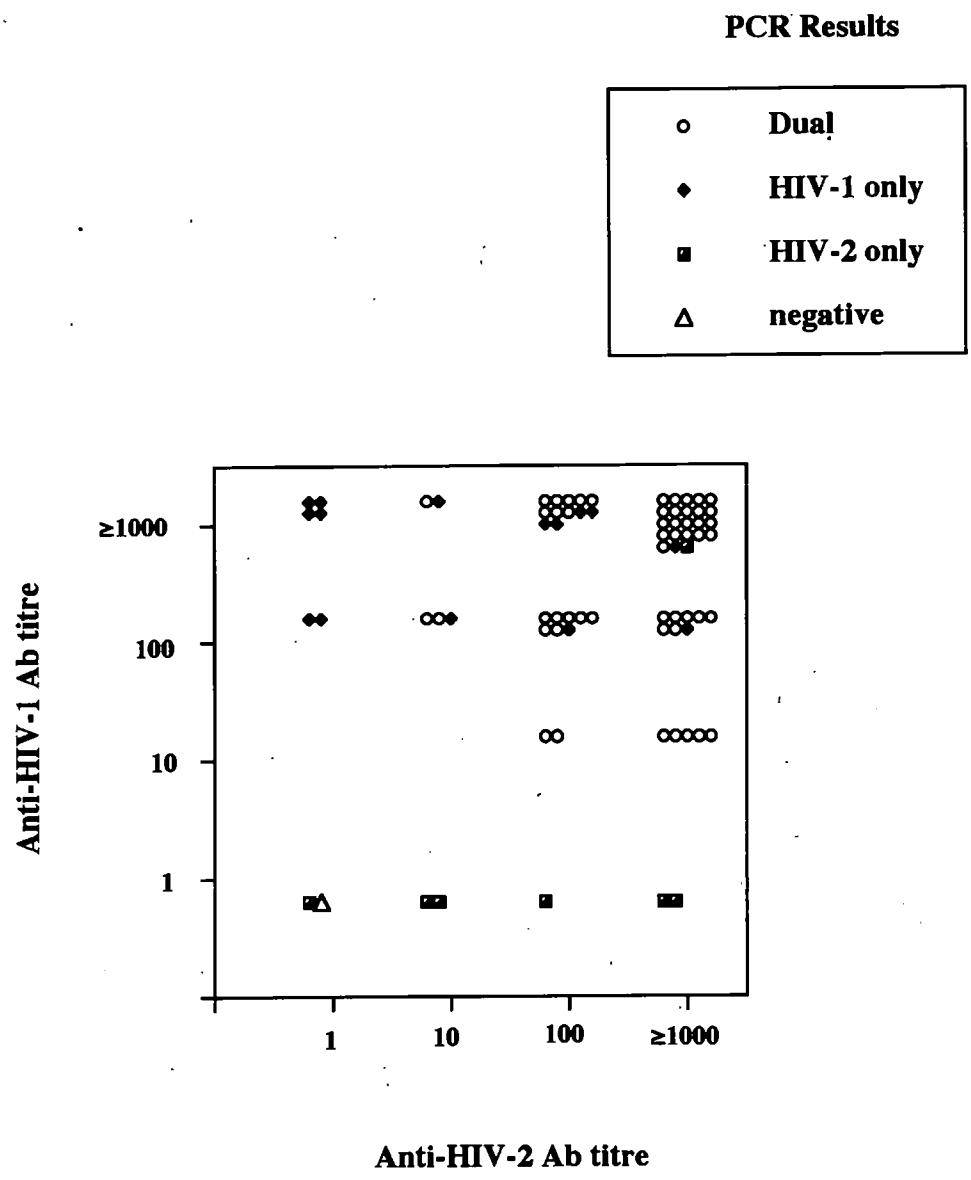

Figure 7.1. Anti-HIV-1 and anti-HIV-2 antibody titres of dually seroreactive subjects according to PCR results.
One subject positive by PCR only for HIV-2 with both anti-HIV-1 and anti-HIV-2 antibody titres of >1000 is subject no. 802 (Table 7.3).

PCR	Hospital-based	Seroconverters	Community-based	Total
HIV-1	6 (13%)	0	0	6 (9.5%)
HIV-2	1*(2%)	0	0	1*(1.5%)
Dual	41** (85%)	9 (100%)	6 (100%)	56** (89%)
Neg	0 (0%)	0	0	0 (0%)
Total	48 (100%)	9 (100%)	6 (100%)	63 (100%)

Table 7.2.
Results of nested PCR in subjects testing dually seropositive by PeptiLav and type specific ELISA at dilution of 1:10.

* This subject (subject 802) tested positive for HIV-1 PCR subsequently (Table 7.3).
** Three subjects with a very low level of HIV-2 provirus were included.

iv. Longitudinal observation of HIV-1 and HIV-2 provirus detection in dually infected individuals

To examine if HIV-2 provirus decreases to undetectable levels after dual seroconversion, HIV-1 and HIV-2 provirus were sequentially tested in 10 subjects who were successfully followed for more than a year. The mean interval between tests was 21 months (range: 15 to 28 months). PCR results, CD4%/CD8% and anti-HIV-1 and anti-HIV-2 antibody titres are shown in Table 7.3. In the majority of subjects, PCR results remained the same but in two subjects the PCR result changed during follow-up: one subject (no 802), in whom HIV-1 provirus was not initially detected, became positive for HIV-1 PCR in the second sample; another subject (no 674) who was initially positive by HIV-2 PCR became negative during the follow-up; this second sample was negative even when tested 10 times.

Subject	date	HIV-1 PCR	HIV-2 PCR	CD4 %	CD8 %	Anti-HIV-1 Ab	anti-HIV-2 Ab
063	Sep. 91	Positive	Positive	42	37	100	>1000
	Dec. 93	Positive	Positive	27	37		
021	Jan. 92	Positive	Positive	25	39	>1000	>1000
	Nov. 94	Positive	Positive	23	43		
802	Jun. 91	**Negative**	Positive	16	74	>1000	>1000
	Sep. 92	Positive	Positive	12	73		
674	Jan. 92	Positive	Positive	16	69	>1000	10
	Apr. 93	Positive	**Negative**	9	70		
123	May 92	Positive	Positive	20	58	>1000	100
	Jan. 94	Positive	Positive	20	46		
080	Aug. 92	Positive	Positive	8	70	100	100
	Aug. 94	Positive	Positive	2	31		
840	Apr. 93	Positive	Positive	6	83	>1000	>1000
	Jun. 95	Positive	Positive	4	81		
220	May 93	Positive	Positive	6	66	>1000	>1000
	Dec. 94	Positive	Positive	3	44		
940	Oct. 93	Positive	Positive	16	63	100	>1000
	May 95	Positive	Positive	17	64		
415	Oct. 93	Positive	Positive	24	64	10	>1000
	May 95	Positive	Positive	28	57		

Table 7.3.
Longitudinal observations of HIV-1 and HIV-2 provirus among 10 dually seropositive subjects.

7.a.3. Discussion.

It was observed that the majority of dually seroreactive individuals were infected with both HIV-1 and HIV-2 as judged by initial PCR screening which has been shown in previous studies. However even with two highly specific antibody assays: competitive ELISA and peptide-based assay and the use of a highly sensitive PCR revealed a proportion of dually seroreactive individuals who were singly positive by PCR. Two explanations for this discrepancy were examined in this study.

Firstly HIV proviral load was thought to be too low to be detected by a single nested PCR test. A nested PCR test able to detect a single molecule (Berry *et al*, 1994), was used to examine $1x10^5$ cells. Thus if the frequency of cells harbouring HIV provirus is less than one in $1x10^5$ cells, a single nested PCR test may fail to detect viral genome. In order to detect the virus at a very low frequency, PCR negative individuals were examined with four more tests and three more subjects were found to have a very low level of HIV-2 proviral DNA. It was also observed that in one patient the level of HIV-2 provirus declined to undetectable levels after 15 months of follow-up, indicating that HIV-2 provirus might occasionally be undetectable despite a genuine mixed infection, provided that the first HIV-2 PCR result was not due to a laboratory contamination. A similar phenomenon has been observed by *in vitro* culture of PBMCs from dually infected individuals in which HIV-2 became undetectable after 6 weeks of culture while HIV-1 remained detectable (Peeters *et al*, 1994). In another patient, HIV-1 provirus was not detected in the first sample but was detected in the second sample. One possible explanation is that the first sample was taken close to the time of superinfection and dual seroconversion and the HIV-1 infection may have only been present at too low a level at the time of sampling.

Secondary antibodies against one type of HIV cross-react with heterotypic viral antigens and often mimic dual seroreactivity. In this study it has been shown that there is a

significant difference in antibody titres between PCR dually positive and PCR singly positive individuals and that even with multiple PCR tests, viral DNA was not detected among those with a low titre, indicating that antibodies with a titre of <10 were mostly likely to be cross-reactive in origin rather than due to superinfection.

Based upon the above observation, after redefining dual seroreactivity as a positive PeptiLav and a positive competitive ELISA for both HIV-1 and HIV-2 at a serum dilution of 10, 56/63 (89%) of dually seroreactive individuals were found to be dually infected by PCR. All 53 PCR dually positive subjects in Table 7.1 fullfilled this new definition. However 6 (9.5%) subjects could not be shown to be dually infected by quadruplicate PCR. Since antibody profiles of these six subjects were indistinguishable from PCR dually positive individuals, it was believed that these individuals were genuinely dually seroreactive as a result of dual infection but that some harboured only an undetectable level of HIV-2.

It was found that when dual seroreactivity was diagnosed using both the competitive ELISA and the peptide-based assay, 18/57 (32%) of hospital-based subjects were positive by PCR for only one HIV type which was mostly HIV-1 whereas 4/10 (40%) of dually seroreactive community-based subjects were positive by PCR only for HIV-2 only (Table 7.1). Thus this data shows that if the less vigorous definition of dual seroreactivity of low stringency is applied, the pattern of PCR results may vary considerably in different study populations. With a more stringent definition for dual seroreactivity which incorporated dilution prior to assaying, this problem was greatly reduced (Table 7.2). For the purpose of seroepidemiological studies, economical and practical methods for serodiagnosis of dual infection are needed. Although titration of HIV type-specific antibody is tedious, this study showed that competitive ELISA at 1/10 dilution was sufficiently accurate as was previously shown (Tedder *et al*, 1988). Various criteria for dual seroreactivity have been

used in different studies (George *et al*, 1992; Peeters *et al*, 1994). Difference in results of comparison between PCR and serology may be due both to a variation of serological diagnostic criteria and also to a difference in study population Careful standardisation of any method to be used remains a necessary procedure.

7.b. Comparison of HIV-1 and HIV-2 proviral load among dually infected individuals

7.b.1. Introduction.

Genomic structures of HIV-1 and HIV-2 are very similar and there is some amino-acid sequence identity. Thus when an individual with one type of HIV is exposed or co-infected with the other type of HIV, significant immunological and virological interactions between the two viruses are expected. A study of a cohort of commercial sex workers (CSWs) in Senegal has suggested that 70-75% of HIV-2 infected individuals are protected from subsequent HIV-1 infection (Travers *et al*, 1995). However the finding was methodologically controversial as selection of HIV-uninfected control CSWs was unclear (Greenberg et al, 1996) and such a gross level of cross-protection has not been found in other cohorts (Aaby et al, 1997; Ariyoshi et al, 1997: supplement to Chapter 7). Nonetheless, there are several reasons to believe that a protective effect may be plausible. Cytotoxic T-lymphocytes (CTL) elicited by one type of HIV cross-reactively recognise the other type of HIV (Nixon et al, 1990; Rowland-Jones et al, 1995; Bertoletti et al, 1997). Neutralising antibodies have been shown to cross-react (Weiss et al, 1988; Bottiger et al, 1990). Some C-C chemokines: MIP1α, MIP1β and RANTES, which can block infection of both HIV-1 and HIV-2 have also been described (Cocchi et al., 1995).

The aim of the second part of this chapter is to study the interaction between HIV-1 and HIV-2 *in vivo* by measuring HIV-1 and HIV-2 proviral load among dually infected individuals.

7.b.2. Results.

i. Study subjects.

Of 56 HIV-1 and HIV-2 PCR dually positive patients identified in the first part of Chapter 7, 34 hospital patients and 8 seroconverters {7 seroconverters from HIV-2 single infection and 1 from HIV-1 single infection} were tested by quantitative PCR. The first part of this chapter also found 7 individuals who were genuinely dually seropositive by a new stringent criteria: both anti-HIV-1 and anti-HIV-2 antibody titres ≥10 but did not have PCR-proven mixed infection. Of those, 5 patients: 4 HIV-1 PCR positive / HIV-2 PCR negative and 1 HIV-1 PCR negative / HIV-2 PCR positive patients who had valid $CD4^+$ cell count data, were also shown in some analysis.

ii. Specificity of HIV-type specific quantitative PCR

In order to evaluate the specificity of HIV-type specific quantitative PCR, 11 HIV-1-infected subjects and 11 HIV-2-infected subjects were tested with HIV-2-specific and HIV-1-specific quantitative PCR respectively. None of HIV-2 singly infected subjects or HIV-1 singly infected subjects tested by heterotypic HIV-quantitative PCR showed any radiometric signal which was higher than the signal of the smallest copy number of positive standard control (3 copies / 10^5 PBMCs).

iii. Comparison of HIV-1 proviral load and HIV-2 proviral load among dually infected individuals.

Geometric means of CD4 adjusted HIV-1 and HIV-2 proviral load were calculated among 42 patients with PCR-proven dual infection (Table 7.4). The geometric mean of HIV-1 proviral load was significantly higher than that of HIV-2 proviral load. When patients were categorised into three CD4 groups, the difference was highly significant only among those with a low CD4% of <14% whereas the viral load was similar among patients with a relatively high CD4% ≥14%.

Five PCR singly positive patients with genuine dual seroactivity by the new stringent criteria, were not included to calculate the geometric means in Table 7.4. However it was noteworthy that the median (range) CD4% of the 5 dually seroreactive patients without PCR-proven mixed infection was significantly lower than the median (range) CD4% of 42 dually seroreactive patients with PCR-proven mixed infection {3.0% (1-17%) versus 18.5% (4-43%) respectively; p=0.017 by Wilcoxon two-sample test}.

HIV-1 proviral load and HIV-2 proviral load in 42 dually infected patients were compared by plotting them in a scatter gram (Figure 7.2.). The correlation between the two types of viral load was not significant ($r = -0.12$, $p>0.1$ by Spearman's test). The figure also showed the viral load of 5 dually seropositive patients without PCR-proven mixed infection, assuming that the other type of HIV was under detectable level by nested PCR but they were not included in the analysis for the correlation. It was noteworthy that none of dually infected patients had both HIV-1 and HIV-2 proviral load lower than 10 copies/10^5 PBMCs.

CD4%	Dually infected patients				HIV-1 infected patients*		HIV-2 infected patients*	
	N	HIV-1 proviral load	HIV-2 proviral load	p value	n	HIV-1 proviral load	n	HIV-2 proviral load
<14%	15	7112 (2241, 22569)	319 (86, 1193)	0.003	22	4068 (2160, 7658)	16	2450 (731, 8211)
14-28%	20	420 (119, 1489)	153 (54, 433)	0.3	20	562 (255, 1241)	10	1916 (664, 5527)
>28%	7	207 (21, 2066)	166 (13, 2035)	0.9	17	120 (52, 273)	23	47 (26, 88)
Total	42	1026 (423, 2483)	202 (97, 419)	0.007				

Table 7.4.
The geometric mean (95% C.I) of proviral load among dually infected patients.
*Proviral load data (copies / 10^5 $CD4^+$ cells) among HIV-1 or HIV-2 singly infected patients who were recruited from the MRC hospital, were derived from another study (Berry et al., manuscript submitted)

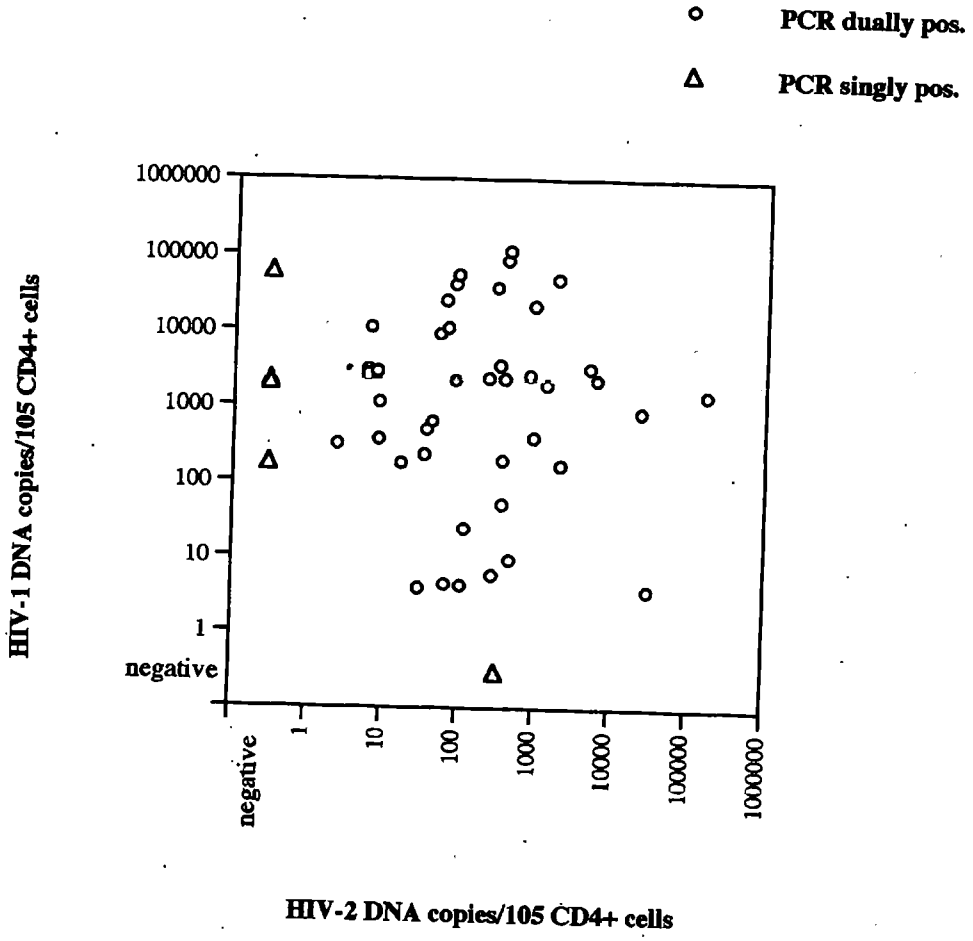

Figure 7.2.
HIV-1 and HIV-2 proviral load among dually infected patients.
Five dually seroreactive patients with both anti-HIV-1 and anti-HIV-2 antibody titres ≥10 but without PCR-proven mixed infection are indicated by open triangles.

iv. Proviral load in relation to $CD4^+$ cell count

CD4 adjusted HIV-1 proviral load was plotted in relation to their CD4% (Figure 7.3). A significant inverse correlation was noticed (r= - 0.47 p=0.002, Spearman's test). On the other hand when CD4 adjusted HIV-2 proviral load was plotted in relation to CD4%, no significant inverse correlation was found (r= - 0.11, p=0) (Figure 7.4.).

The figures also show the viral load of 5 dually seropositive patients without PCR-proven mixed infection but the analysis for the correlation did not include the five PCR singly positive patients.

v. HIV-proviral load in subjects who became dually infected during follow-up.

The mean of $CD4^+$ cell count and the geometric means of HIV-1 and HIV-2 DNA viral load among known converters were compared with the other patients (Table 7.5). The mean CD4% among converters from HIV-2 single infection to dual infection was significantly higher than that among the other hospital patients (p=0.01). The geometric mean HIV-2 DNA viral load was similar between converters and the other hospital patients. The geometric mean of HIV-1 DNA viral load among converters from HIV-2 single infection was lower than that among the other hospital patients but after adjusting for CD4%, there was no difference between the two groups (p=0.5). The five dually seropositive patients without PCR-proven mixed infection were not included in this analysis.

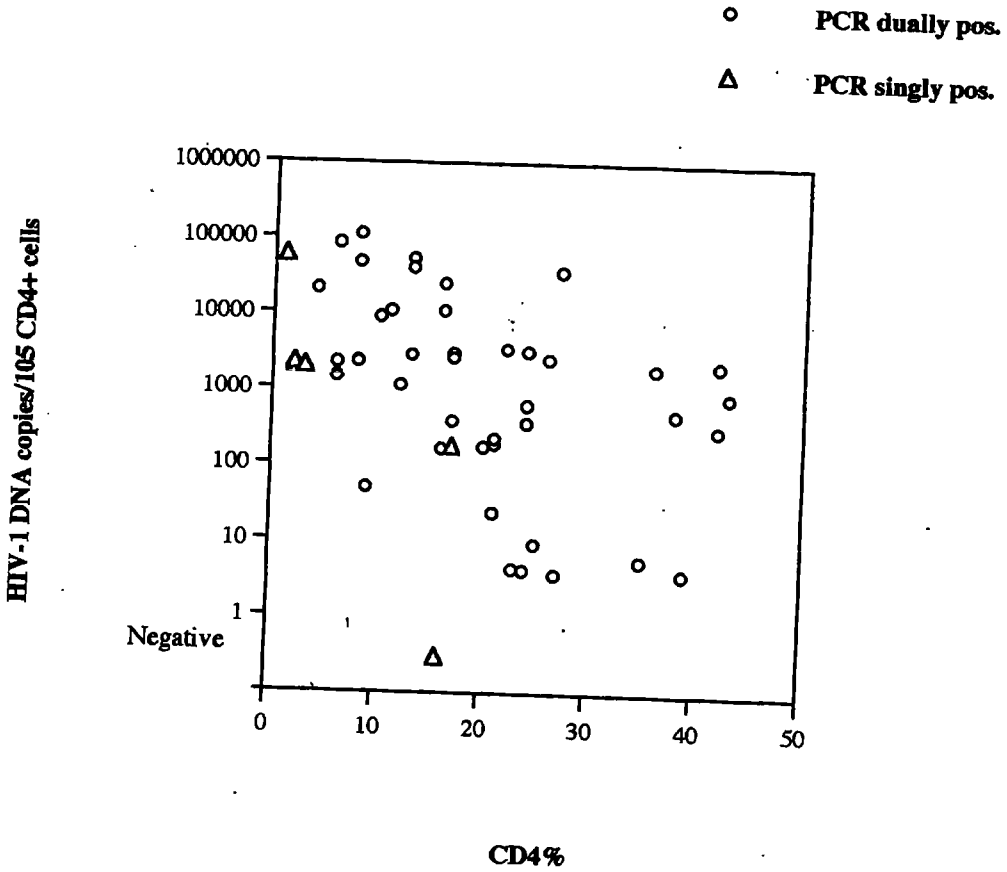

Figure 7.3.
HIV-1 proviral load and CD4% in dually infected individuals.
Five dually seroreactive patients without PCR-proven mixed infection are indicated by open triangles.

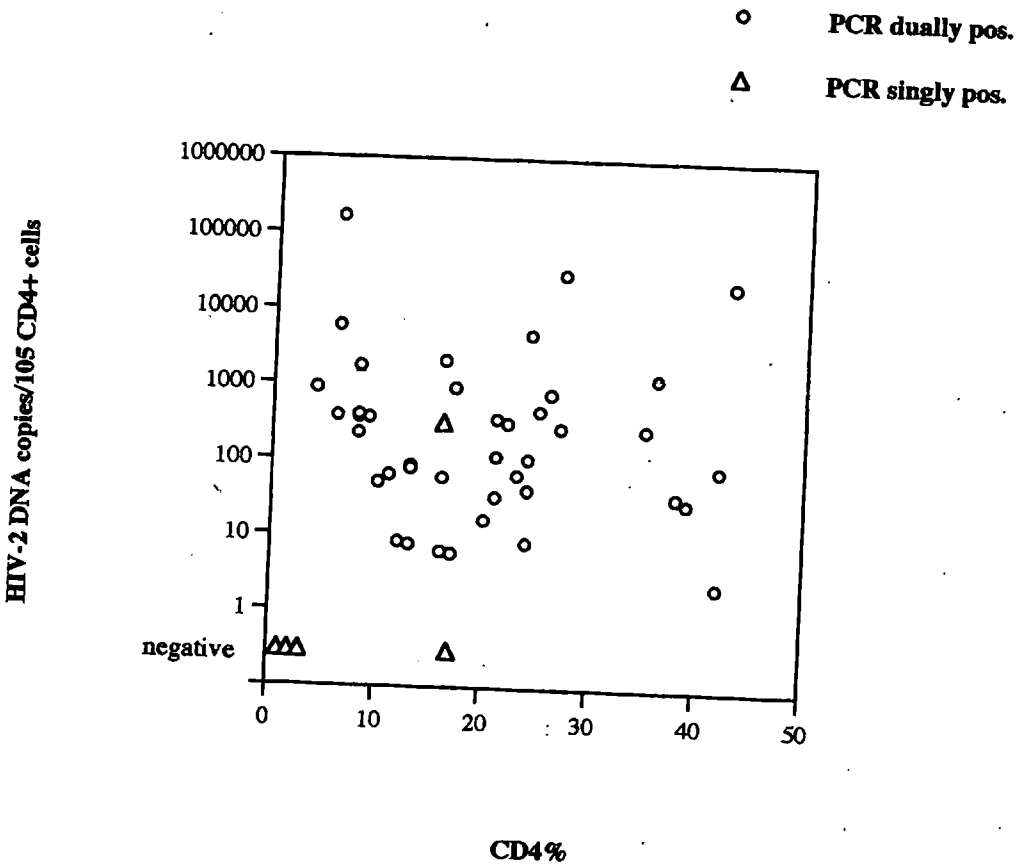

Figure 7.4.
HIV-2 proviral load and CD4% in dually infected individuals.
Five dually seroreactive patients without PCR-proven mixed infection are indicated by open triangles.

	CD4 (%)	*HIV-1 proviral load	*HIV-2 proviral load
Seroconverters			
HIV-1 to dual	38	486	36
(n=1)	-	-	-
HIV-2 to dual	27.4	191	112
(n=7)	(19.9, 35.0)	(16, 2334)	(27, 469)
Dually infected	17.9	1483	239
hospital patients	(14.2, 21.6)	(533, 3966)	(100, 573)
(n=34)			

Table 7.5.
The geometric mean of proviral load among seroconverters and the hospital patients.
* Proviral copies/10^5 $CD4^+$ cells (95% CI).

7.b.3. Discussion.

One of the main objectives of the second part of HIV-1/HIV-2 dual infection study is to see if pre-existing HIV-2 protective immunity is sufficiently cross-reactive to suppress the growth of the superinfecting HIV-1 and to prevent from HIV-disease progression. It was shown that when macaques pre-infected with HIV-2 were challenged with diverse strains of pathogenic SIVsm, although they were not protected from SIV infection, they were protected from SIV-disease progression by reducing SIV viral load (Putkonen *et al.* 1995). Therefore cross-reactive protective immunity elicited by preceding HIV-2 infection may not confer compete protection against HIV-1 infection. However like in the HIV-2/SIV model, the cross-reactive protective immunity may suppress an initial surge of super-infecting HIV-1 during the HIV-1 primary infection more efficiently than HIV-uninfected individuals, and as a consequence dually infected individuals have a lower level of HIV-1 provirus than HIV-1 singly infected individuals. In order to test this hypothesis, HIV-1 proviral load among dually infected patients was compared with previous data of HIV-1 proviral load among HIV-1 singly infected patients (Berry *et al.* manuscript submitted) (Table 7.4). The comparison was made possible since the HIV-1 quantitative PCR assay used for the previous study was essentially same as for this chapter and the study subjects for the both studies were recruited from the same cohort in The Gambia. There was not any significant difference in the geometric means of HIV-1 proviral load between dually infected patients and HIV-1 singly infected patients in any of CD4 groups ($p>0.1$). Therefore this observation does not support the initial hypothesis. Several reasons were thought for this lack of evidence of cross-protection. Firstly the proportion of HIV-2 infected individuals who have cross-protection may be so small that the difference was not demonstrated by the simple overall comparison of proviral load. It is noteworthy that there were four subjects whose HIV-1 proviral load was relatively low of <10 copies / 10^5 $CD4^+$ despite an abnormally low CD4% <29%. The presence of cross-protection may be restricted by limited alleles of class I and/or class II MHC molecules. HLA typing of whole

dually infected individuals is warranted. Secondly in 34 dually infected subjects it was not known if they were converted to dual infection from HIV-1 or HIV-2 single infection. HIV-1 proviral load may not be low if dually infected individuals were converted from HIV-1 single infection. However as it was discussed in Chapter 1, epidemiological data indicate that HIV-1 epidemic started later than HIV-2, suggesting the majority of dually infected individuals were converted from HIV-2 single infection. In addition, the mean HIV-1 proviral load of the eight converters from HIV-2 single infection was not significantly different from that of the other dually infected patients (Table 7.5). Thirdly the replication of both viruses may be mutually enhanced thus the partial cross-protective effect may have been cancelled out. Antagonistic CTL by cross-reactive CTL was also thought as a mechanism for the mutual enhancement (Klenerman et al., 1994; Meier et al., 1995). This possibility has recently been tested by Rowland-Jones et al., and B27-restricted cross-reactive CTL was not found to induce antagonism (Rowland-Jones et al., manuscript submitted). Virologically Tat and Rev of one type of HIV can act upon the other type of provirus, though HIV-2 Tat and HIV-2 Rev act on HIV-1 TAR and RRE less efficiently than HIV-1 Tat and HIV-1 Rev act on HIV-2 TAR and RRE (Chapter 1). Although a positive correlation was not found between HIV-1 and HIV-2 proviral load among dually infected patients, without RNA viral load data one can not conclude that there is no mutual stimulation of HIV replication. Further investigation of HIV-1 and HIV-2 RNA viral load in dually infected individuals is necessary.

The other main finding in the second part of this chapter is that dually infected individuals with a low CD4% had a significantly lower HIV-2 proviral load than HIV-1 proviral load. When HIV-2 proviral load was also compared with HIV-2 proviral load data among HIV-2 singly infected individuals in the previous study (Berry et al., manuscript submitted) (Table 7.4), it was found that the level of HIV-2 provirus in dually infected individuals was significantly lower than that in HIV-2 singly infected individuals among the groups with a

CD4%<14% and 14-28% (p=0.033 and p=0.01 respectively). Intriguingly the four dually seroreactive patients with negative PCR results for HIV-2 provirus also had a low CD4% (Figure 7.4). Therefore it is plausible that these four patients had a very low level of HIV-2 provirus which was not detectable. Mechanisms for the lower HIV-2 proviral load among dually infected individuals in advanced disease have been thought. First, the EBV-specific CTL precursor frequencies are known to be maintained in advanced HIV-1 infection while HIV-1-specific CTL activity selectively deteriorates (Carmichael *et al.* 1993). Thus in this group of patients, HIV-2-specific CTL may maintain. Second, since HIV-1 and HIV-2 use common co-receptors such as CCR3, CCR5 and CXCR4, in addition to CD4 molecule (Brön *et al* 1997), the target cell population is likely to be shared by the two viruses. As HIV-1 Nef and Vpu are known to down-regulate surface expression of CD4 molecules (Garcia et al., 1991; Aiken et al., 1994; Kerkau et al., 1997), cells infected with HIV-1 may become resistant to the superinfection (Benson et al., 1993). Thus competition of infecting a limited number of target cells may become apparent among those with a few number of $CD4^+$ cells and fast-growing HIV-1 may dominate the target cells. Third, immunosuppression in the dually infected individuals is mainly caused by HIV-1 with rapid/high replication capacity while slow/low replication capacity of HIV-2 may remain.

Chapter 8

General discussion

8.a. The role of HIV-2 viral load in the pathogenesis of HIV-2 infection.

From the extensive studies of HIV-1 viral load, it is now widely believed that viral load is important in the pathogenesis of AIDS as the turn-over rate of HIV-1 productively infected cells is directly related to the rate of $CD4^+$ T-cell loss. Thus patients with a high rate of HIV-1 replication *in vivo*, which is more closely reflected by the high level of HIV-1 RNA in plasma than proviral DNA in lymphocytes, have a rapid turn-over of HIV-1-productively infected cells which results in a decline in $CD4^+$ T-cell count. This thesis has shown that the burden of HIV-2 virus is also an important determinant for the pathogenesis of HIV-2-induced AIDS for the level of HIV-2 RNA in plasma, in particular, correlates with a rate of $CD4^+$ T cell decline and with death. Therefore like HIV-1 infection, the rate of $CD4^+$ T-cell loss is likely to be directly related to the rate of turn-over of HIV-2 productively infected cells.

8.b. Why do HIV-2-infected individuals live for long?

One of the main aims in this thesis has been to measure HIV-2 viral load in order to answer the central question why HIV-2-infected individuals live longer than HIV-1-infected individuals. However findings in Chapter 3 raised a paradox as the level of HIV-2 proviral DNA in healthy individuals living in a rural village in Guinea-Bissau was high and more or less equivalent to that of the provirus in asymptomatic HIV-1 subjects as reported in published papers. However in that study, HIV-2 proviral load was not compared with the level of HIV-1 proviral DNA in African population living in the same environment. Berry *et al.*, have recently compared the proviral load of HIV-1 and HIV-2 patients attending the MRC hospital and again found that there was no significant difference in proviral DNA load after adjusting for their $CD4^+$ cell count (Berry et al., manuscript submitted). Thus the conclusion is that HIV-2-infected individuals live longer not because they have

successfully eradicated the virus but because they are able to live with a substantial amount of HIV-2 provirus. The same study showed that the most obvious difference between the two infections was that RNA viral load of HIV-2 patients with a high $CD4^+$ cell count was much lower than that of similar HIV-1 infected patients (Berry et al., manuscript submitted). Interestingly the difference in the mortality between HIV-1 and HIV-2-infected patients attending the hospital was significant only in the group of patients who had a normal $CD4^+$ cell count at entry to the study but not in the other groups of patients (S Jaffar, personal communication). Thus HIV-2-infected individuals in the early phase of disease have a disproportionally high level of proviral DNA in relation to of RNA as compared with HIV-1-infected individuals. Although a proper comparison between HIV-1 and HIV-2 viral load should be made using community-based patients before reaching a final conclusion, the low level of HIV-2 RNA, but not HIV-2 DNA, is likely to be the main mechanism for the better prognosis of HIV-2 infection.

8.c. Why is the level of HIV-2 RNA virus in plasma low?

The next question is why the level of HIV-2 RNA virus in plasma is low despite a substantial level of HIV-2 DNA. Using the model postulated in Chapter 1, two possibilities may explain the lower level of HIV-2 RNA in plasma. One is that if the production of HIV-2 virions per cell is as high as in HIV-1 infection and if the half life of productively infected cell is similarly short, the size of productively infected cell pool must be small (Figure 8.1). The other explanation is that if the production of HIV-2 virions per cell is low and the half life of productively infected cell is long, the size of the productively infected cell pool may be large (Figure 8.2). In both cases the number of cells dying would be small and the level of RNA in plasma low. A study which measures the frequency of HIV-2-infected cells expressing mRNA may give more insight into the mechanisms of the low level of HIV-2 RNA in plasma. Other crucial questions which need answering in order to understand HIV-2 virus population dynamics are the proportion of HIV-2 proviruses which

are replication-competent, which types of cells harbour HIV-2 proviruses, and the half life of HIV-2-productively infected cells. HIV-2 population dynamics may differ significantly from HIV-1 population dynamics.

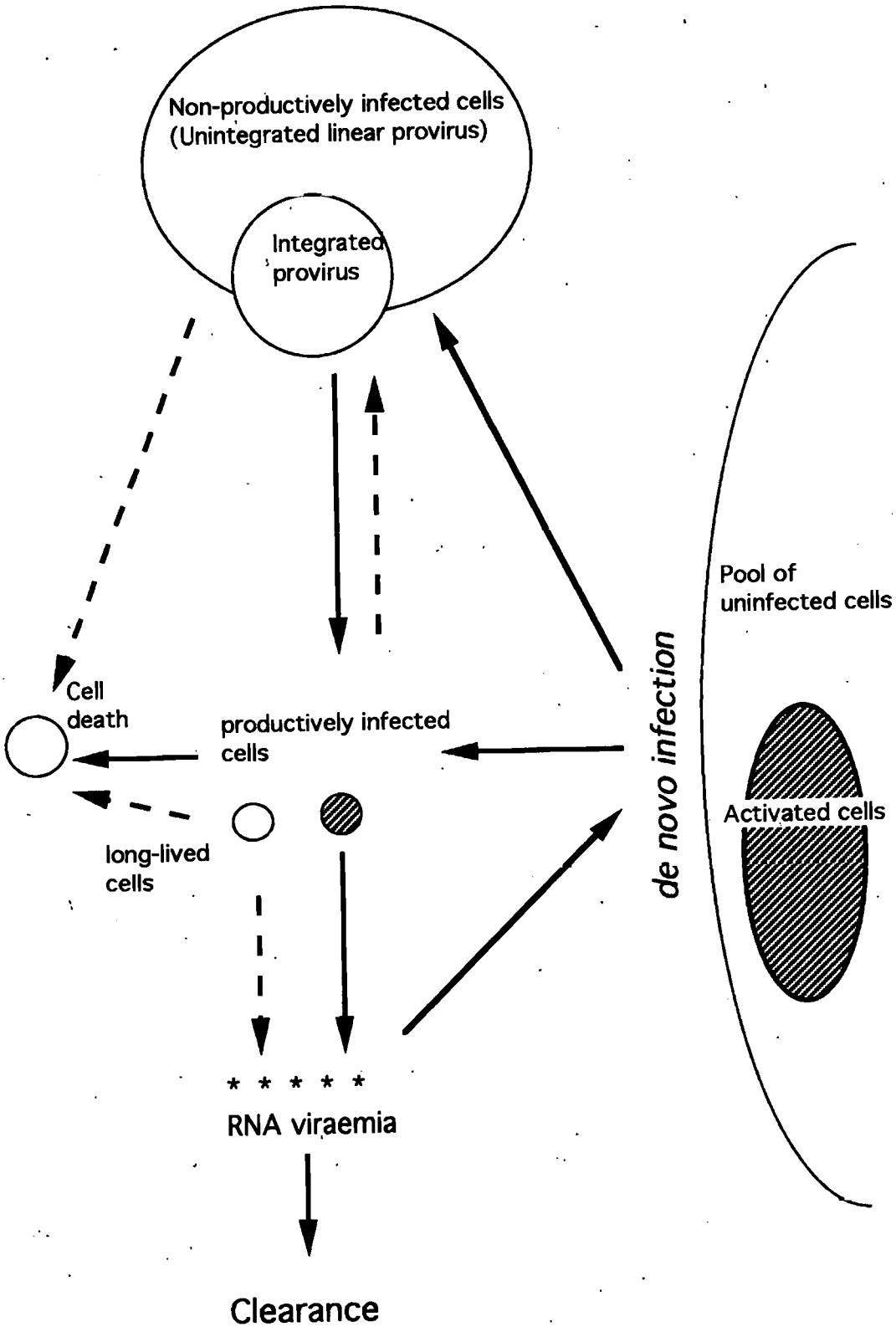

Figure 8.1. HIV-2 polulation dynamics 1 in vivo.
The production of HIV-2 virions per cell is high and the size of the productively infected cell pool is small.

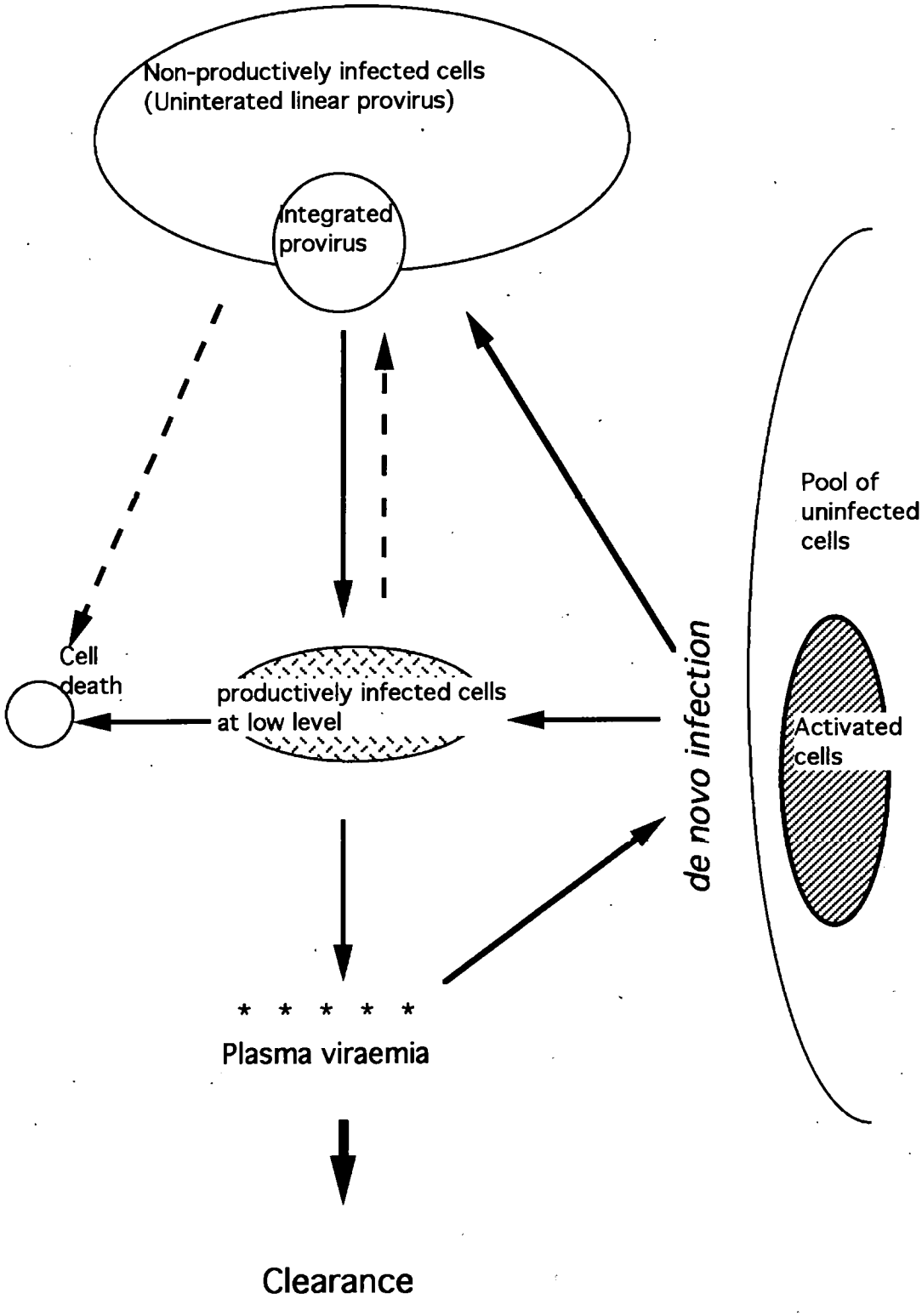

Figure 8.2. HIV-2 population dynamics 2 in vivo.
The production of HIV-2 virions per cell is low and the size of productivel infected cell pool is large.

8.d. What factor determines the low HIV-2 RNA viral load?

During acute infection, HIV-1-infected individuals have a high level of viraemia before the seroconversion and in the majority of patients the level of virus in plasma declines sharply, coinciding with the development of HIV-specific host-immune responses such as CTL but not with neutralising antibodies (Ariyoshi et al., 1992; Koup et al., 1994; Borrow et al., 1994; Connick et al., 1996). Then the viral load appears to become stabilised at a certain set point (Mellors et al., 1995). Therefore viral load after the acute infection is a reflection of the balance between the ability of the host-immune response to control viral replication and the replicative capability of the virus under the immune pressure. Although in HIV-2 infection, acute HIV-2 viraemia has not been described, it is plausible that a similar phenomenon occurs during the acute phase of HIV-2 infection and that the level of HIV-2 RNA is likely to be determined by the balance between the host's immunity and the replicative capability of the virus.

i. Host-factors determining the low level of HIV-2 viral replication.

The human host may develop a better immune response against HIV-2 than HIV-1. The role of host factors is supported by the findings in Chapter 5 that a strong HIV-2-specific CTL activity in asymptomatic HIV-2 patients has been found and there is an inverse correlation between HIV-2 proviral load and CTL activity. HIV-2-specific CTL activities have also been demonstrated in fresh PBMC (Gotch et al., 1993) and frequently cross-recognise HIV-1 antigen (Rowland-Jones et al., 1995; Bertoletti et al., 1998). In addition, neutralising antibodies (NA) against autologous viruses have been readily demonstrated in HIV-2-infected individuals (Bjorling et al., 1993). One explanation for the efficient immune response to HIV-2 is that HIV-2 antigen may be presented more efficiently or more conserved regions may be presented by HIV-2 as compared with HIV-1. The recent finding that the Vpu protein down-regulates the surface expression of MHC class I molecule, is interesting as the protein does not exist in HIV-2 (Kerkau et al., 1997). HIV-1

Nef has also been suggested to be important as a mechanism for evading the immune system as it down-regulates the expression of the MHC class I molecule (Schwartz et al., 1996) and up-regulate Fas-Ligand (Xu et al., 1997). It is, therefore, important to find if HIV-2 Nef has a similar function or not.

Alternatively the viruses intrinsically replicate at a low level with the result that escape mutants emerge infrequently and CTL and NA are effective.

ii. Viral factors determining the low rate of HIV-2 replication.

The lower rate of viral replication may be a results of intrinsic factor in the virus. If the host immunity is the major factor determining the low HIV-2 viral load, HIV-2 isolates from an infected individual should grow to a high titre in *in vitro* when co-cultured with donor cells from HIV-naive individuals where host-immune suppression no longer takes place. Limited information suggests that this is not the case for even after several passages the virus remains at low titre, suggesting intrinsic factors are also important in regulating viral replication (Evans et al., 1988; Kong et al., 1988; Albert et al., 1990). However it remains unknown if the dominant population of slow/low viruses which are usually found in HIV-2-infected asymptomatic individuals, is due to a selective advantage of such viruses. Within HIV-2 viruses, the slow/low viruses may be recognised by the immune system less efficiently than the rapid/high viruses as the slow/low viruses present less viral antigen than the rapid/high viruses. Therefore it is difficult to discuss host and viral factors separately.

The interaction between host cellular factors and enhancers in the LTR region is an important factor which determines the rate of HIV replication. As discussed in Chapter 1, there are substantial differences in the structure of enhancers of the HIV-1 and HIV-2 LTR regions and thus cellular factors, such as TNF, activate HIV-2 LTR differently from HIV-1 LTR. Therefore HIV-2 may be less efficiently enhanced by various co-infection than HIV-

1; this may be one reason why HIV-2 RNA viral load is low. In this thesis, the interactions of HIV-2 with HTLV-I and malaria were investigated. Although there was no evidence that co-infection with HTLV-I enhanced HIV-2 proviral load, a small increase in HIV-2 viral load was noticed among individuals with malaria parasitaemia (Chapter 6); the trend may be more obvious in HIV-1 infection, however in this study only one HIV-1 infection with malaria parasitaemia was observed. It would be interesting to do a similar study in HIV-1 infection. More studies to compare the enhancing effects of various co-infections on HIV-1 and HIV-2 replication are needed.

The other important factor determining the rate of HIV-2 replication is the characteristics of the susceptible host cell population. The observation that HIV-2 primary isolates use a wider range of chemokine receptors is intriguing since it suggests that the susceptible cell population may be different between HIV-1 and HIV-2 (Bron et al., 1997; Heredia et al., 1997; McKnight et al., manuscript submitted). Currently HIV-2 envelope glycoprotein has been found to bind to CD8 as well as to CD4 molecules on human T cells (Kaneko et al., 1997). If cell population susceptible to HIV-2 is less activated than that of HIV-1, this would explain the smaller size of productively infected cell population. The study of HIV-2 infected cell population *in vivo* is warranted.

Although the reasons for the lower transmission rates of HIV-2 are not addressed in this thesis, it is plausible that the lower HIV-2 viral load may relate to the low rate of transmission since in HIV-1 infection maternal RNA viral load appears to be associated with vertical transmission (Fang et al., 1995; Dickover et al., 1996). The data presented in this thesis supports the hypothesis that the lower infectivity of HIV-2 may be due to the lower rate of HIV-2 virus replication resulting in a lower viral load.

8.e. Other possible explanation for the better prognosis of HIV-2 infection

The African Green monkey is the natural host for SIV_{agm}. The virus does not cause immunodeficiency in this host although SIVagm replicates at a substantial rate (Hartung et al. 1991). It remains unknown if some strains of HIV-2 replicate to a high titre without killing infected cells as does SIV_{agm} in its natural host. The turnover of the HIV-2-productively infected cells as compared with the turn-over rate of HIV-1-productively infected cells has not been investigated. This question can be answered by comparing HIV-1 and HIV-2 viral kinetics in patients receiving potent anti-retroviral drugs. An alternative approach is to conduct a survival analysis of HIV-1 or HIV-2-infected patients who are matched for base-line $CD4^+$ cell count and base-line RNA viral load.

8.f. Summary

The host-virus relations in HIV-1 and HIV-2 infections are compared and summarised in Table 8.1. The conclusion is that in general HIV-2-infected individuals live longer than HIV-1 infected individuals not because they have eradicated the virus but because they are able to tolerate a substantial level of HIV-2 provirus which does not replicate as fast as HIV-1. This low steady-state in HIV-2 infection is established either because the HIV-2 virus intrinsically grows at low rate or because the human host can develop efficient immune response which controls HIV-2 viral replication or both. Possible research areas are summarised in Table 8.2. The main reason for this low steady-state relationship remains largely unknown.

	HIV-1 infection		HIV-2 infection
RNA viral load	+++	>>	+
DNA viral load	++	=	++
Biological properties	rapid/high > slow/low		slow/low > rapid/high
Intra-patient variability	High	>	low?
Efficient CTL	++	<	+++?
Autologous NA	Low	<	high?
Escape mutant (CTL)	Frequent	>	less frequent?
Escape mutant (NA)	Frequent	>	less frequent?

Table 8.1. Summary of host-virus relations in HIV-1 and HIV-2 infections.

Research fields	Hypothesis
Host factors	
Neutralising antibody epitopes and its escape mutant	NA epitopes in HIV-2 are more conserved
CTL epitopes and its escape mutants	CTL epitopes in HIV-2 are more conserved
Viral factors	
HIV-2 virus kinetics	A half life of HIV-2 productively infected cells is long.
LTR functions of HIV-2 slow/low viruses	HIV-2 LTRs are less responsive to various cellular factors
Co-receptor usage of HIV-2 slow/low viruses and cell population infected with HIV-2 provirus	HIV-2 uses different co-receptors and infects different cell populations
Nef function of HIV-2 slow/low viruses	HIV-2 Nef does not down-regulates surface CD4 and MHC class I molecule expression and does not up-regulates Fas ligand.

Table 8.2. Summary of important research areas of host and viral factors in HIV-2 infection.

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Appendix 1

Supplement to Chapter 7

Does HIV-2 infection provide cross-protection against HIV-1 infection?

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Does HIV-2 infection provide cross-protection against HIV-1 infection?

A study of prostitutes in Senegal suggests that HIV-2 infection confers 75% protection against subsequent HIV-1 infection [1]. There are several reasons to believe that such a protective effect may be plausible, as cross-reactive cytotoxic T-lymphocytes (CTL) between HIV-1 and HIV-2 [2,3], cross-reactive neutralizing antibodies [4,5], and C-C chemokines that can block infection of both HIV-1 and HIV-2 [6] have been described.

The Gambia is one of the very few countries where HIV-2 has been present since before the HIV-1 epidemic started [7]. To confirm the hypothesis that HIV-2 protects against HIV-1 infection we have retrospectively analysed HIV-1 incidence data from 504 initially seronegative and 87 HIV-2-seropositive commercial sex workers (CSW) in The Gambia, who were seen at the Medical Research Council (MRC) clinic between June 1988 and July 1995. Sera were tested twice or more in 189 seronegative and 53 HIV-2-seropositive subjects by a HIV type-specific competitive enzyme-linked immunosorbent assay (Murex Diagnostics, Dartford, Kent, UK) and a peptide-based immunoenzymatic strip method (PeptiLAV; Diagnostic Pasteur, Marnes-la-Coquette, France). Dual infection was confirmed by nested polymerase chain reaction with HIV type-specific long terminal repeat (LTR) primers (kindly provided by Dr N. Berry, University College London, London, UK). Mean age, the proportion of CSW with active syphilis at recruitment, and the incidence of culture-proven gonorrhoea did not significantly differ between the two groups (Table 1; $P > 0.1$); however, the incidence rate of HIV-1 infection among HIV-2-positive CSW was 8.7 per 100 person-years of observation (PYO), being higher than that of HIV-negative CSW (5.4 per 100 PYO). The rate ratio of 1.7 (95% confidence interval, 0.7–3.9) suggests a slightly increased risk for HIV-2-infected women. The observed HIV-1 incidences in our cohort are strikingly higher than those in the Senegalese study (1.1 per 100 PYO in HIV-2-infected women and 2.5 per 100 PYO in HIV-negative women) [1]. Although our data did not allow adjustment for unsafe sexual practices, it does not support the hypothesis that there is substantial protection against HIV-1 infection by preceding HIV-2 infection.

The apparent difference between the Travers study [1] and our study suggests that it may be difficult to consistently demonstrate evidence of cross-protection by HIV-2 against HIV-1. Epidemiological factors such as the level of exposure to HIV-1 may have varied between the two studies. Biological factors may also vary, and experiments with monkeys suggest that there is a window period between infection with attenuated viruses and subsequent protection against challenge with pathogenic simian immunodeficiency virus [8]. Thus, the duration of preceding HIV-2 infection may influence the interaction of HIV-1 and HIV-2 infections. If protection did exist and was mediated mainly by CTL, only some HIV-2-infected subjects with certain human leukocyte antigen (HLA) alleles such as HLA-B27 and HLA-B35, which cross-reactively recognize HIV-1 [2,3], may be protected by preceding HIV-2 infection. Susceptibility of HIV-1 may also vary according to the genotype of the infecting HIV-1 virus, and finally, an undetermined proportion of HIV-negative CSW in HIV-2 endemic areas may be resistant to HIV infection, as has been observed in Nairobi [9]. In conclusion, we emphasize that further epidemiological, virological and immunological studies are required to elucidate how the two types of HIV interact.

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Table 1. Characteristics of initially HIV-2-positive and HIV-negative commercial sex workers.

Group	n	Mean (SD) years of age	Proportion with active syphilis at enrolment (95% CI)	PYO	No. HIV-1 infections	IR (95% CI) HIV-1	IR (95% CI) Gonorrhoea
HIV-2-positive	53	29.2 (5.4)	0.33 (0.20–0.46)	92.0	8	8.7 (4.3–17.4)	8.9 (4.9–17.8)
HIV negative	189	28.2 (5.7)	0.24 (0.18–0.30)	369.2	19	5.1 (3.3–8.1)	9.5 (6.8–13.2)
Rate ratio						1.7 (0.7–3.9)	0.9 (0.4–2.0)

Incidence rates (IR) per 100 person-years of observation (PYO) of gonorrhoea and HIV-1, and the resulting rate ratios are shown; active syphilis was diagnosed if both *Treponeme pallidum* haemagglutin assay and rapid plasma reagin tests were positive. CI, Confidence interval.

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Appendix 2

Publications arising from the studies presented in this thesis.

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K Ariyoshi, F Cham, N Berry, E Harding, S Sabally, PT N'Gom, K Ishikawa, S Jaffar, T Corrah, R Tedder, H Whittle. Diagnosis of HIV-1/-2 dual infection using dilutional analysis of type specific antibody. manuscript in preparation.

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K Ariyoshi, F Cham, N Berry, A Wilkins, P Aaby, O Larsen, O Jobe, PT N'Gom, S Jaffar, F Dias, RS Tedder, H Whittle. No enhancement of Human Immunodeficiency Virus Type-

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